University of Glasgow

Debbri, Hawa Abdulgader (1996) *Plant extracts as treatment for diabetes mellitus.* PhD thesis.

http://theses.gla.ac.uk/2113/

Copyright and moral rights for this thesis are retained by the author

A copy can be downloaded for personal non-commercial research or study, without prior permission or charge

This thesis cannot be reproduced or quoted extensively from without first obtaining permission in writing from the Author

The content must not be changed in any way or sold commercially in any format or medium without the formal permission of the Author

When referring to this work, full bibliographic details including the author, title, awarding institution and date of the thesis must be given

Glasgow Theses Service
http://theses.gla.ac.uk/
theses@gla.ac.uk

PLANT EXTRACTS AS TREATMENT FOR DIABETES MELLITUS

A thesis submitted for the degree of Doctor of Philosophy in the Faculty of Medicine, University of Glasgow.

By

Hawa Abdulgader Debbri

Institute of Biomedical and Life Science, West Medical Building and Department of Pharmacology

University of Glasgow

July 1996

ACKNOWLEDGMENTS

I would like to extend my sincere thanks to Dr. Paul Skett my supervisor, who provided excellent advice, encouragement and guidance throughout these three very short years.

I am extremely grateful to my family particularly to my dad and my brother Yossef, who encourage me to do it, and who supported me in all ways they could throughout my stay at Glasgow. Without their help, patience and unfailing moral and practical support throughout my period of study, this work would not have been possible.

I wish to thank Professor T. Stone and Dr. D. Pollock, for kindly allowing me to carryout this research in their department.

Furthermore, I am deeply indebted to the staff at the Department of Diabetes, Al-Afia Hospital, Libya particularly, Dr. Ahmed (Medical Director) for offering the facilities for the clinical aspects of this project.

I will seize this opportunity to thank Mr. Ahmed Saleh for collecting the plant samples from the desert, and Mr. Saleh Al-fitouri for sending it.

I am also very grateful to my mum taken the pain to separate the aerial parts of the plant and drying them. I wish to express my gratitude to the patients who volunteered the clinical trials and showed keen interest in the results.

I acknowledge financial support from the Libyan Interest Section.

Thanks to Professor J. Connolly for grinding the plant and assisting with the extractions and Dr. J. H. Dickson for the identification of the plants

I would like to express my thanks to the members of the academic (past, present and temporary), technical and secretarial staff in the Department of Pharmacology, particularly to Robert Auld and Adam Ritchie.

Finally, I wish to thank all my colleagues, Naima, Salma, Shamas and Elspeth. I wish them happiness in the future, Dr. Ali Nasr and his wife Titoha for helping me in the last three months of my study, And many thanks to Eric for his help and generous technical assistance.

TABLE OF CONTENTS

Summary:

1-The herbal extract of *Artemisia* has been regarded to be anti-hyperglycaemic since olden times and is commonly used by diabetics in Libya. The present work was designed to evaluate, test and determine which fraction or component of the herb had the hypoglycaemic effects in normal and streptozotocin-induced diabetic rats.

2-The plant extract was administered to the animals in their drinking water and body weight, food and fluid intake and urine volume were all monitored daily. Food and fluid intake and body weight gain in normal rats were not altered by treatment with the plant extract but there was a rise in the urine glucose in the first six rats but rats 7, 8 and 9 were not affected by treatment with plant. Urine volume was increased in all rats suggesting *Artemisia judaica* is a mild diuretic.

3- The streptozotocin-induced diabetic rat model, used in this study, was associated with the characteristic diabetic symptoms of hyperphagia, hyperglycaemia, polydipsia, weight loss and urinary glucose excretion. When a crude aqueous extract of *Artemisia* was given in their drinking water, it had little effect on these symptoms after 10 days of treatment. Urine glucose was reduced in the last two days and ketones in the urine were abolished by this treatment.

4- Diabetes mellitus is known to affect many and varied parameters in rat liver. Insulin, biguanides and sulphonylureas are known antidiabetic diabetic treatments. *Artemisia judaica* extract was tested for its effect on hepatic steroid metabolism and glycogen phosphorylase a activity in comparison with the above drugs. Clearly *Artemisia* does act as an insulin-mimetic in these assays by reversing all the effects produced by the administration of streptozotocin. In particular the changes in the enzyme activities of cytochrome P-450 (2E1, 2B and 2C) on androst-4-ene-3,17-dione metabolism are all reversed by the administration of *Artemisia* extract to diabetic rats. The addition of insulin and/or *Artemisia* extract directly to normal rat hepatocytes, however, did not have any effect on the metabolism of androst-4-ene-3,17-dione. This is in contrast to the results of Hussin & Skett (1988). Extracts of other plants, *Anvillea*

and *Marrubium*, similarly showed little effect on steroid metabolism in isolated hepatocytes.

5- Insulin is known to inhibit glycogen breakdown by decreasing the activity of glycogen phosphorylase *a* and counteracting the stimulatory effect of glucagon (van de Werve and Jeanrenaud, 1987). In this study the effects of insulin on glycogen phosphorylase *a* activity were confirmed as was the reported stimulatory effect of glucagon on the same enzyme. All of the plant extracts mimicked insulin in decreasing glycogen phosphorylase *a* activity. *Marrubium* and *Anvillea* were more potent than *Artemisia*. *Artemisia*, *Marrubium* and *Anvillea* when added together with insulin decreased the activity of glycogen phosphorylase *a* more than extracts alone and they antagonised the effect of glucagon.

6- A start was made in separating the active ingredient(s) from the *Artemisia judaica* extract. From the results of the aqueous and ethanolic extracts of *Artemisia*, it is suggested that the extract contains at least two active ingredients. One which mimicked and enhanced the effect of insulin (in the water extract) and the other (in the ethanol extract) which antagonised the effect of glucagon.

7- The preliminary results of human studies showed that *Artemisia judaica* had a potential use in treating patients with diabetes and it is suggested that *Artemisia judaica* contains material capable of reducing an elevated blood sugar and improving diabetic symptoms.

8- It can be concluded therefore from the results of the present study that *Artemisia* can exhibit significant anti-diabetic effects *in-vivo* and *in-vitro* and has an insulin-like action. *Marrubium* and *Anvillea* when tested on glycogen phosphorylase a have the same action as insulin but these were not further tested due to lack of time. These findings tend to endorse the wide use of these plants in Arab folk medicine and show a great potential for the treatment of diabetes.

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ADP	Adenosine-5'-diphosphate
AMP	Adenosine-5'-monophosphate
ATP	Adenosine-5'-triphospate
$[Ca^{2+}]_i$	Intracellular free calcium ion concentration
Cyclic AMP	Cyclic adenosine 3',5'-monophosphate
D.P.M	Disintegrations per minute
DTT	DL-Dithiothreitol
EDTA	Ethylene diaminetetraacetic acid
EGTA	Ethylene glycol-bis (β- aminoethyl ether),N,N,N,N-tetra-acetic acid
g	Centrifugal g-force
G-6-P	Glucose-6-phosphate
Glc1P	Glucose-1-phosphate
HEPES	N-[2-hydroxyethyl]piperazine-N'[2 ethanesulfonic acid]
I.U	International unit
IDDM	Insulin- dependent diabetes mellitus
INT	Iodonitrotetrazolium
INTH	Iodonitrotetrazolium reduced

kDa	Kilodalton
M.W.	Molecular weight
MOPS	3-[N-Morpholino]propane sulphonic acid
NADP	Nicotinamide adenine dinucleotide phosphate (oxidized).
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NIDDM	Non-insulin-dependent diabetes mellitus
OHase	Hydroxylase
OHSD	Oxosteroid oxidoreductase
PMS	Phenazine methosulphate
PMSH	Phenazine methosulphate reduced
Protein kinase A	Cyclic AMP-dependent protein kinase
S.D.	Standard deviation
Ser	Serine
STZ	Streptozotocin
T.L.C	Thin layer chromatography
U	Unit

INTRODUCTION

1. BACKGROUND:

1.1 Diabetes mellitus :

Diabetes mellitus may be defined as a disorder of metabolism in which a relative or absolute deficiency or lack of effect of insulin leads to chronic hyperglycaemia with or without glucosuria.

Diabetes has probably been known to medical science longer than any other ailment, yet, in many respects it is still poorly understood. It is characterized by hyperglycaemia (high blood glucose) and occurs because the liver and skeletal muscle cannot store glycogen and the tissues are unable to utilize glucose.

There are two main forms of diabetes mellitus; type 1 and type 2.

Type 1 diabetes, also known as insulin dependent diabetes (IDDM), is due to an inadequate insulin secretion resulting from a large decrease in the number of beta-cells in the islets of Langerhans (Eisenbarth, 1986) and Type 2 diabetes, or non-insulin dependent diabetes mellitus (NIDDM), is due to a lack of insulin action in target tissues (Lillioja *et al.*, 1988) and / or insulin resistance that leads to impaired tissue glucose uptake and impaired suppression of hepatic glucose production (Martin *et al.*, 1991). IDDM is usually managed by injection of insulin and NIDDM may be controlled by dietary means such as weight loss and diet control, However, around 50% of NIDDM patients cannot achieve satisfactory control through diet alone and require treatment with a class of drugs collectively referred to as oral hypoglycaemic agents (OHA) (Kennedy *et al.*, 1988)

Most of the pathological conditions of diabetes mellitus can be attributed to one of the following three major effects of insulin lack (1)- Decreased utilization of glucose by the body cells, with a resultant increase in blood glucose concentration (2)-Markedly increased mobilization of fats from the fat storage areas causing deposition of lipids in the vascular walls due to abnormal fat metabolism and resulting atherosclerosis and abnormal breakdown of fats leading to the formation of ketone bodies (3)- Depletion of protein in the tissues of the body.

The overall effect is dehydration of the extracellular space, which then causes dehydration of the intracellular spaces as well (Bowman & Rand, 1983). Glucose

absorbed into the blood stream cannot be utilized by the body's cells due to the lack of insulin, and consequently blood glucose levels rise. In an effort to redress the situation, glucose flows out in the urine as the kidney threshold for glucose is exceeded and excess glucose cannot be reabsorbed. Glucose acts as an osmotic diuretic and large quantities of water are excreted as the glucosuria worsens. This makes the individual thirsty and the patient drinks large volumes of fluid (Watkinson *et al.*, 1993). There is also an increased appetite without any weight gain which is particularly evident in IDDM where patients have a tendency to lose weight.

Also, in type 1 diabetes, the excess breakdown of fat leads to the formation of ketones bodies and organic acids leading to acidosis (Bloom *et al.*, 1985). The liver is supplied with massive amounts of free fatty acids (FFA). These are oxidized in the liver to provide the energy required for glucose synthesis, simultaneously releasing large quantities of ketone bodies into the circulation. At the same time release of amino acids from breakdown of protein or lactate production in peripheral tissues supplies the liver with the carbon substrates essential for glucose formation. This process, which can be a useful source of both glucose and ketone bodies for energy in times of starvation, accelerates hyperglycaemia and ketoacidosis in severe insulin deficiency of juvenile onset (type 1) diabetes. This ketoacidosis only occurs in IDDM individuals, where there is a severe insulin deficiency.

Acute deficiency of insulin, therefore causes a metabolic acidosis (ketosis) due to the shift from carbohydrate to fat metabolism in diabetes (due to the absence of insulin this occurs even normally between meals when secretion of insulin is minimal, but it becomes extreme in diabetes when secretion of insulin is almost zero). When the body depends almost entirely on the fat for energy, the level of ketone bodies in the body fluid rises mainly derived from the ongoing activity of the liver, which releases them into the circulation for transport to the heart, skeletal muscle, and other tissues to be used as an energy source. In the normal fed state, the concentration of ketone bodies is relatively low because insulin stimulates the synthesis of fatty acids, a competing pathway for the use of acetyl CoA. Insulin also inhibits lipolysis, thereby decreasing the supply of fatty acids, which are a major source of acetyl CoA in the liver. Thus, when insulin concentrations are decreased, as occurs in type 1 diabetes or fasting, production of ketone bodies is favoured. Severe ketosis does not develop in normal individuals

because only a small amount of insulin is needed to inhibit lipolysis in adipose tissue (Espinal, 1989)

NIDDM usually develops after 35 years of age and most diabetics of this type are obese, and the hormone is often present in plasma at near-normal or even above-normal levels, and therapy often does not require administration of insulin (Luciano, 1990). Indeed, the presence of insulin and the ability of sulphonylureas to evoke release of the hormone are indicative of NIDDM. Because only a small amount of insulin is needed to prevent ketone body formation, the non-insulin-dependent diabetic rarely develops ketosis. In some cases of NIDDM the plasma insulin concentrations are actually higher than normal due to hyporesponsiveness to insulin, termed insulin resistance. This is explained by the fact that the target cells are relatively insensitive to the hormone. In such resistant cells, a higher concentration of insulin is needed to elicit a response than in normal cells. In some cases of insulin resistance the concentration of insulin receptors appears to be decreased (Home, 1989).

The sequence of events coupling obesity to a diminished number of insulin receptors is shown (Figure 1.1). Because insulin secretion is increased during food absorption, any person diabetic or not who chronically overeats secretes, an average, increased amounts of insulin. Over time, the resulting elevation of plasma insulin induces a reduction in the number of insulin receptors. Thus insulin itself is responsible for the decrease in target-cell responsiveness (insulin resistance), producing a higher plasma glucose concentration at any given plasma insulin level. The tendency toward a higher plasma glucose concentration is small in non-diabetic overeaters because the islet cells respond by secreting enough additional insulin to get the job done despite the reduction in available receptors. In contrast, the diabetes-prone person may also secrete additional insulin but not enough to prevent significant hyperglycaemia

Diabetic coma is rarely seen in non-insulin-dependent diabetics, presumably because their endogenous insulin prevents ketosis. However, a related condition, referred to as hyperosmolar coma, can occur. It is most often observed in elderly individuals and is usually preceded by an illness or other stressful situation that increases the requirement for insulin. Under these circumstances, the insulin present becomes insufficient to prevent glucosuria and excess water loss leading to a hyperosmolar plasma.

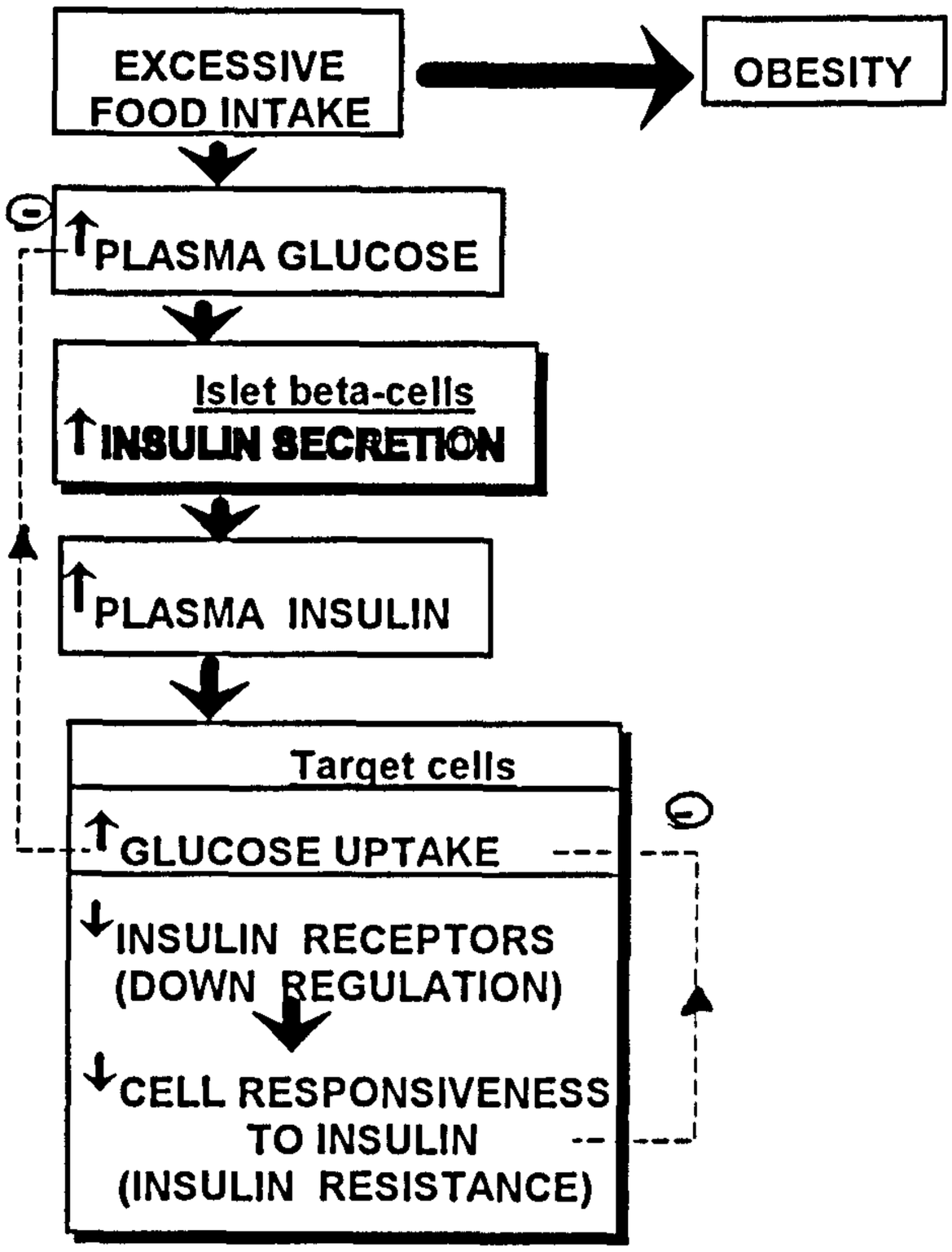


Figure 1.1 Postulated mechanisms by which chronic overeating leads to chronically elevated plasma insulin and diminished cell responsiveness to insulin (Luciano, 1986).

Fluid loss is compounded when vomiting is associated with the precipitating illness. As dehydration become severe, urinary output decreases in spite of the high urinary levels of glucose. Thus renal excretion of glucose falls, and blood sugar and serum osmolarity increase to extremely high levels, leading to loss of consciousness. Like diabetic coma, hyperosmolar coma is life threatening and is a particular grave condition in older diabetics, who may already have compromised cardiovascular function (Joslin, 1989).

Diabetes mellitus therefore affects protein, lipid and carbohydrate metabolism and reflects a state of impaired insulin function. The net effect of insulin in the body is to increase metabolic energy store by promoting the storage of protein, lipid and carbohydrates (Ganong, 1989). Thus when insulin function is impaired, hyperglycaemia results from the reduction in glucose uptake and increased rate of gluconeogenesis and hyperlipidaemia results from increased lipase activity and reduced deposition of triglycerides. It is these dysfunctions that produce much of the pathology associated with diabetes mellitus. Diabetics are 25 times more prone to blindness, suffer from an increased risk of myocardial infarction and increased incidence of peripheral vascular disease such as gangrene. They are also more prone to renal failure and to peripheral neuropathy than non-diabetics.

1.2 Treatments of Diabetes Mellitus :

Type 1 (IDDM) diabetics lack insulin and their treatment is hormone replacement therapy with insulin. Type 2 (NIDDM) diabetics, however; often do not respond to insulin and require alternative drugs if dietary control is insufficient.

There are two main classes of oral hypoglycaemic drugs, the sulphonylureas such as tolbutamide and glibenclamide, and the biguanides such as phenformin and metformin. Tolbutamide and phenformin were introduced in clinical practice in 1954, and 1957 respectively. Tolbutamide is still widely used though phenformin has been withdrawn since 1982 as a result of the risk of lactic acidosis, (Oates *et al* 1981). However metformin which belongs to the same class as phenformin is the drug of choice in the therapy of obese NIDDM diabetes. The use of sulphonylureas, the other

class of hypoglycaemic agents, is also not without its problems and side effects. They have adverse effects including coma (Ferner and Neil, 1988) particularly in the elderly and patient with impaired hepatic or renal function who are taking longer acting sulfonylureas.

Sulfonylureas and metformin are valuable treatments for hyperglycaemia in NIDDM, but they are often unable to lower glucose concentrations to within the normal range, or to reinstate a normal pattern of glucose homeostasis (Lebovitz, 1985; Bailey & Nattrass, 1988). Even insulin therapy does not reinstate a normal pattern of glucose homeostasis in most NIDDM patients, and over vigorous insulin treatment may carry an increased risk of atherogenesis and hypoglycaemia (Peacock & Tattarsall, 1984).

As a result of these numerous side effects of oral hypoglycaemic drugs, there has been an increase in research of other potential oral hypoglycaemics and renewed for attention to alternative medicines and natural therapies. This has stimulated a new wave of research in traditional practices and the World Health Organization Expert Committee on Diabetes has listed these as one of the treatment for diabetes which should be further investigated (Watt & Wood, 1988; WHO Expert Committee, 1980).

Traditional oral hypoglycaemic plants might provide a useful source of new oral hypoglycaemic compounds for development as pharmaceutical entities, or as simple dietary adjuncts to existing therapies. The known use of plants for diabetes dates from the Ebers papyrus of about 1550BC and many traditional plant treatments for diabetes are used throughout the world (Bailey & Day, 1989). After the introduction of insulin therapy, the use of traditional treatments for diabetes greatly declined in societies, although some plant extracts are still used as prophylactics and adjuncts to conventional medicine.

For this purpose the natives of Libya use a hot water decoction made from the whole plants, *Artemisia Judaic , Anvillea carcinii and Marrubium vulgare*. There appears to be no documented reports on the antidiabetic activity of *Artemisia judaica, Marrubium vulgare*, and *Anvillea carcinii*, although there is a report on *Artemisia abyssinia* which indicates that it has hypoglycemic activity (Mossa, 1985). Extracts of *Artemisia judaica* have been shown to have effects on the heart (digitalis-like) (Gala *et al.*, 1974) and on the isolated guinea-pig ileum (Abdalla & Zarga 1987). *Artemisia herba alba* has also been used by the local populations of some Middle East countries as

an anthelminthic (Khaffagy *et al.*, 1988), and also it has antidiabetic (Husni & Al-Badr, 1988, Farjou *et al.*, 1987, 1988, Al-Kazraji *et al.*, 1993) and antibacterial (Hussein-Ayuob 1990) actions. In 1986, Abdalla, found a parasympathomimetic compound in a crude extract of *Artemisia judaica* which stimulated muscarinic receptors and also certain anticancer agents have been found (Alwan *et al.*, 1989). Artemisinin, santonin, judaicin, and cirsimaritin have been isolated from *Artemisia* sp (Khaffagy & Tosson, 1968).

In this study the anti-diabetic properties of the plants *Artemisia judaica, Anvillea carcinii and Marrubium vulgare* L are investigated. These plants will be discussed in detail later. It is the aim of the project to validate scientifically the use of these plants in the treatment of diabetes. These plants have been used successfully in the treatments of diabetes in Libya as well as other Arabic speaking countries but they have not received much in the way of medical or scientific scrutiny.

1.3 Glycogen Phosphorylase

1.3.1 Function and Regulation:

Glycogen phosphorylase plays a central role in the mobilization of carbohydrate in a wide variety of organisms including bacteria, fungi, plants and insects. It catalyses the first step of the intracellular degradation of glycogen, where inorganic phosphate (Pi) is used in the cleavage of an [$\alpha 1 \rightarrow 4$] glucosidic bond to yield glucose 1-phosphate according to the equation.

$$(\alpha\text{-1,4-glucoside})_n + P_i \leftrightarrow (\alpha\text{-1,4-glucose})_{n-1} + \alpha\text{-D-glucose 1-phosphate (Glc1}P\text{)}$$

Glycogen phosphorylase catalyses both the forward and the reverse reaction. Under *in-vivo* conditions the phosphate concentration is usually so high that the catalyzed reaction is in the direction of glycogen degradation.

Glycogen phosphorylase can exist in two forms, *a* and *b*. Inactive phosphorylase *b* can be activated in two ways, namely (a) by covalent alteration through phosphorylation of ser-14 at its terminal tail (giving glycogen phosphorylase *a* ;GPH*a*) in response to hormone and neural signals, and (b) allosteric binding to the activator AMP.

1.3.2 Role of Insulin and Glucagon in control of glycogen metabolism:

In the transition between the fed state and periods of short fasting, the net hepatic glucose output is determined by the reciprocal activities of glycogen synthase and glycogen phosphorylase. The regulation of the activation and inactivation of both of these enzymes has been the object of intense investigation, particularly with regard to the roles of various hormones, including insulin and glucagon.

Insulin has also been implicated as a major regulator of hepatic glucose output. Hers (1976) demonstrated that in the isolated perfused liver, insulin acts to decrease hepatic glucose output. Miller and Larner (1973) have reported that insulin acts to promote glycogen synthesis by a direct activation of glycogen synthase. Witters *et al.* (1978) found that both glucose and insulin are major regulators of hepatic glycogen synthase and glycogen phosphorylase in hepatocytes isolated from fed rats. In 1967, Bishop *et al.* demonstrated that insulin administered *in-vivo* activates dog liver glycogen synthase and inactivates phosphorylase. Glucagon reverses these changes.

Several studies have suggested that control of glycogen metabolism in liver is regulated by a cascade enzyme system that is similar to the one already established in skeletal muscle (Krebs, 1962; Hers, 1961). Glucagon stimulates the formation of active phosphorylase *a* (Sutherland & Robinson, 1951) and converts glycogen synthase from an active form into an inactive form (Stalman & Hers, 1971). Insulin on the other hand, converts glycogen synthase from an inactive form into an active form, both *in-vivo* and *in-vitro* (Bishop & Larner, 1967; Kreutner & Goldberg, 1967; DeWulf & Hers, 1968). There is general agreement that insulin antagonizes the effect of glucagon on glycogen metabolism (Beesj *et al.*, 1995). Miller and Larner (1973) concluded, after examining the effect of insulin on hepatic glycogen synthase by use of either diabetic or glucagon-stimulated normal rats, that insulin increases the formation of glycogen synthase with a

concomitant decrease in cyclic AMP-dependent glycogen synthase kinase activity. Insulin appears to interfere with glucagon action by reducing the elevated levels of cAMP (Blackmore *et al* 1979).

1.4 Pancreatic Hormones:

1.4.1 Insulin:

An increase in blood glucose signals the release of insulin from the beta cells of the pancreas. Insulin circulates in the blood, serving as first messenger to inform several tissues that excess glucose is present. Insulin receptors, located on the plasma membranes of insulin-responsive cells, respond to insulin binding by producing secondary messengers that mediate insulin action and promote glucose use within these tissues. The pancreas responds to a decrease in blood glucose with less release of insulin but greater release of glucagon (Harris, 1992). Insulin increases glucose utilization rates in part by promoting glycogenesis and inhibiting glycogenolysis in liver (Figure 1.2). Insulin promotes glycogen accumulation in liver by activating glycogen synthase and inactivating glycogen phosphorylase (Harris, 1992). Insulin is known to inhibit glycogen breakdown by decreasing the activity of glycogen phosphorylase *a* (van de Werve & Jeanrenaud, 1987).

1.4.1.1 Mechanism of action of insulin

The enzymatic pathway involved in glycogen synthesis and breakdown has been characterized in most mammalian tissues. Glycogen synthesis in liver is, at least partially, controlled by the activity of glycogen synthase, the enzyme responsible for catalyzing the incorporation of glucose, via uridine diphosphoglucose, into glycogen (Letoir & Cardini, 1957). The effect of insulin is to promote the activation of glycogen synthase from a glucose-6-P-dependent to a glucose-6-P-independent form. Witter *et al.* (1978) have reported that insulin acts to promote glycogen synthesis by a direct

activation of glycogen synthase (Figure 1.2). It has become widely accepted that insulin promotes the arrest of glycogenolysis and the deposition of glycogen in the liver. A partial inactivation of glycogen phosphorylase has been observed, although inconsistently, in anaesthetized rats (Stalmans *et al.*, 1974) and much more systematically in rhesus monkeys (Curnow *et al.*, 1975).

Thus by measurement of glycogen alone, it appears that the current concepts of insulin action at the biochemical level are unable to explain all of the effects observed *in-vivo*. Insulin could act by lowering the hepatic concentration of cyclic AMP. Such an effect can be readily demonstrated with isolated liver preparations when the concentration of the nucleotide has been previously increased by glucagon or adrenaline (Exton *et al.*, 1971). Whereas the inactivation of phosphorylase was a constant feature following the administration of insulin, the activation of glycogen synthase occurred only occasionally. The activation of synthase was only observed when phosphorylase activity had reached a very low value (Stalmans *et al.*, 1975). The activity of glycogen phosphorylase and synthase is controlled to a great extent by phosphorylation, which in turn is catalysed by the cAMP-dependent protein kinase (protein kinase A). Thus changes in the level of cAMP by insulin may have a marked effect on the activity of the glycogen phosphorylase and synthase. The same pattern of reciprocal changes in phosphorylase and synthase has been noted in a study with anaesthetized mice treated with glucose (Stalmans *et al.*, 1974). Insulin has also been implicated as a major regulator of hepatic glucose output. The studies of Witter & Avrub (1978) demonstrated that, in the isolated perfused liver, insulin acts to decrease hepatic glucose output. Glycogen phosphorylase activity may, therefore, be a better measure for insulin-like activity than glycogen synthase or glycogen concentration alone.

1.4.2 Glucagon

Even though insulin plays a central role in controlling the metabolic adjustments between the absorptive and post-absorptive states, the secreted product of pancreatic islet A-cells, glucagon, is also very important. It seems that glucagon secreting A-cells and insulin secreting β-cells, as a coupled endocrine system, is a major factor in the regulation of fuel metabolites.

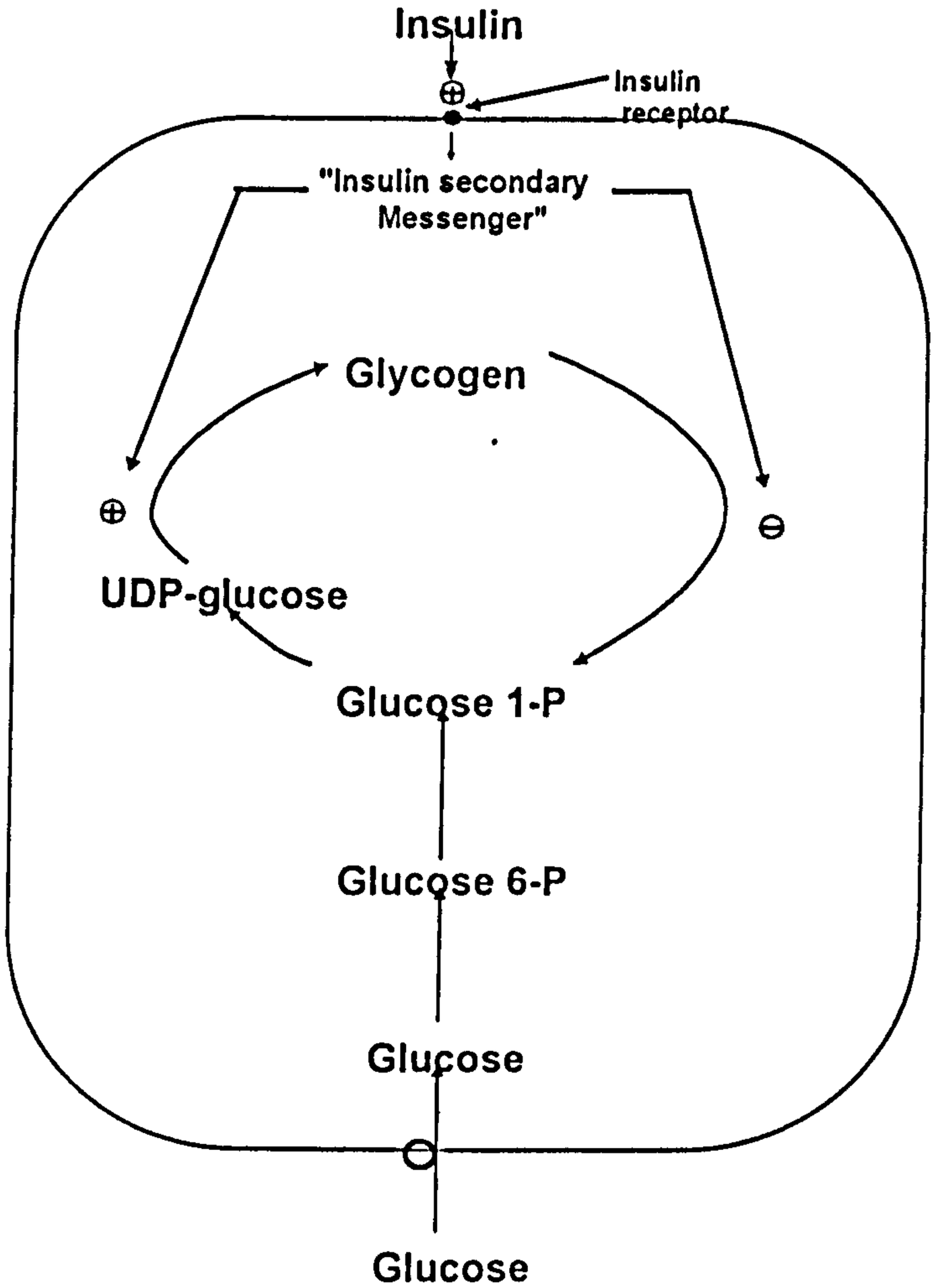


Figure 1.2 Insulin acts via secondary mediators of its action to inhibit glycogenolysis and promote glycogen synthesis in liver (adapted from Harris, 1992).

Glucagon has many effects on the same metabolic processes that are influenced by insulin. It has the opposite effect to insulin and again the liver is the major site of action of glucagon.

1.4.2.1 Mechanism of action of glucagon

Glucagon is released from the A-cells of the pancreas in response to low glucose levels in the blood. One of glucagon's primary jobs during periods of low food intake (fasting or starvation) is to mobilize liver glycogen, that is, stimulate glycogenolysis, in order to ensure that adequate blood glucose is available to meet the needs of glucose-dependent tissues. Glucagon circulates in the blood until it interacts with glucagon receptors such as those located on the plasma membrane of liver cells (Figure 1.3). Binding of glucagon to these receptors activates adenylate cyclase and triggers the cascades that result in activation of glycogen phosphorylase and inactivation of glycogen synthase (Harris, 1992).

1.5 Cytochrome P-450:

Cytochrome *P*-450 initially was reported in 1958 by Klingenberg & Garfinkel. They noticed the presence of a carbon monoxide-binding pigment in rat liver microsomes. The pigment displayed a maximal absorbance at a wavelength of 450 nm upon bubbling carbon monoxide into a dithionite reduced rat liver microsomal suspension and thus, it was called P(pigment)-450. In 1964 Omura & Sato published spectral evidence that this pigment was a haemoprotein, a new b-type cytochrome. Cytochrome P-450 plays an important role in the metabolism of endogenous substrates, as well as exogenous xenobiotics. The biological effectiveness is responsible for the hydroxylation or oxidative dealkylation of various drugs by liver microsomes (Cooper *et al.*, 1965).

The cytochromes P-450 are a group of haemoproteins and are located in almost every tissue, and in many subcellular membrane fractions including the

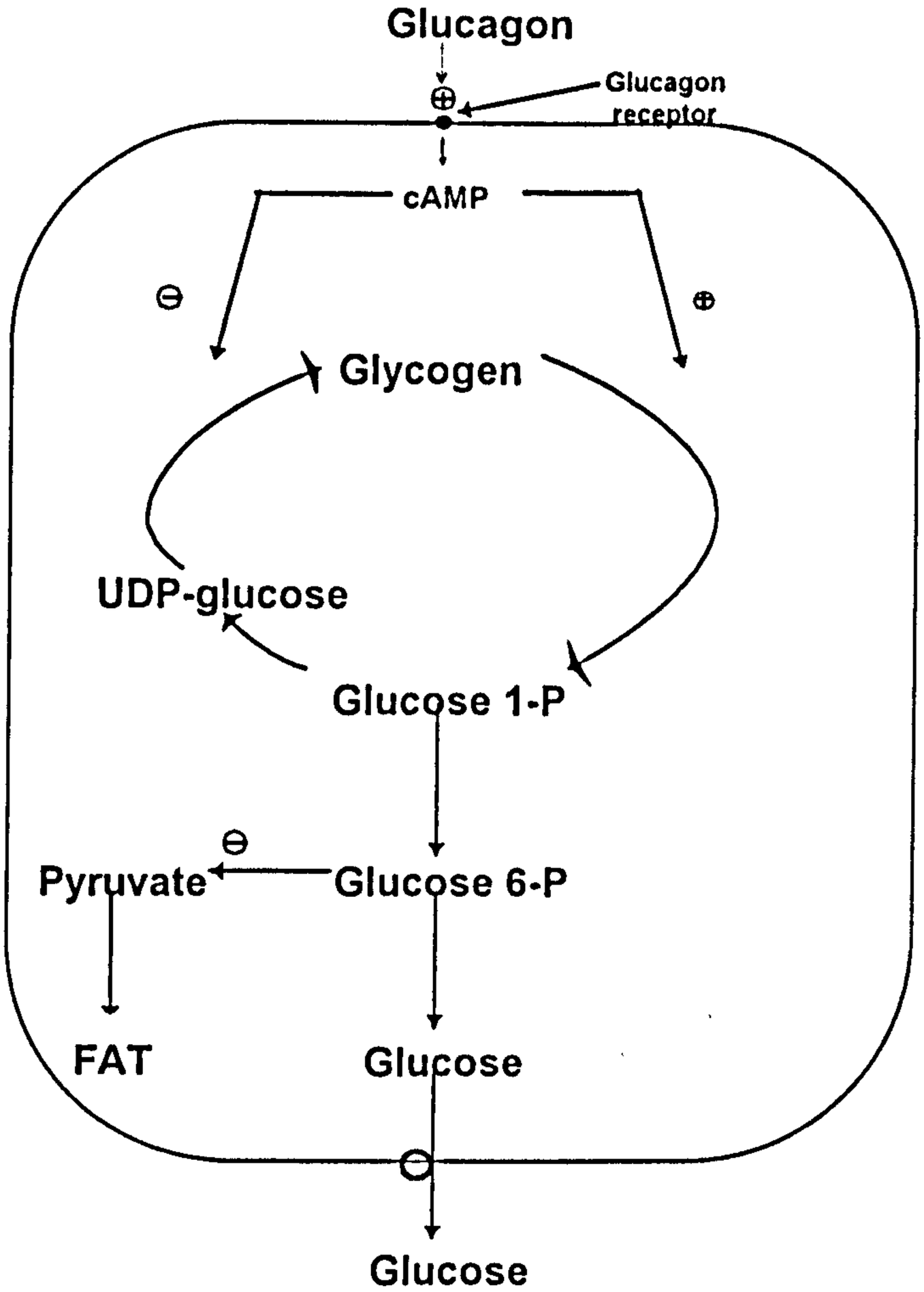


Figure 1.3 Glucagon acts via secondary mediators of its action to activate glycogenolysis and inhibit glycogen synthesis in liver (adapted from Harris, 1992)

endoplasmic reticulum (Brodie *et al.*, 1955) and the mitochondria of the adrenal gland (Harding *et al.*, 1964).

In mammals, cytochrome P-450 is found at varying concentration in microsomes from liver, kidney, small intestine, lung, adrenal cortex, skin, testis, placenta, and other tissues (Omura & Sato 1964). The parenchymal cells of liver (hepatocytes) contain the highest concentration of cytochrome P-450 although it has also been demonstrated that the non parenchymal cells of the liver also have the capacity, though lower than the parenchymal cells, to metabolize a wide range of drugs and chemicals (Steinberg *et al.*, 1976). Hepatocytes contain different constitutive forms of cytochrome P-450, some of these forms of cytochrome P-450 varying in content on challenge of the animal with xenobiotics. Other forms appear to respond to homeostatic control signals varying with conditions in the body, from developmental changes to sexual development, and in response to pathophysiological conditions such as starvation, diabetes and hypertension. These changes all result in differential abilities of the animal to metabolize drug and xenobiotic chemicals as well as endogenous compounds such as fatty acids and steroids (Schenkman, 1990). The cytochrome P-450 mono-oxygenases play a central role in the biotransformation of xenobiotics (including drugs, insecticides, pesticides, food additives, products in smoke and pyrolysis products produced by cooking) into more water soluble products that permit detoxication and removal from the body and metabolism of certain xenobiotics such as benzene, acetoaminophen, halothane and nitrosamines, to more reactive products that are toxic and / or carcinogenic (Defeng *et al.*, 1993).

The finding of the successful stabilization of cytochrome P-450 by glycerol (Ichikawa & Yamano, 1967) permitted solubilization and purification experiments on cytochrome *P*-450 leading to the understanding that there is a large family of these proteins with different substrate specificity. The metabolism of xenobiotics is affected by many factors (Paine, 1981, Gibson & Skett, 1994), one of them being hormones. It has been established that the alteration in drug and steroid metabolism may be attributed to endocrinological disorders and the pancreas is one whose secretions affect xenobiotic metabolism.

Insulin and glucagon secreted by the islets of Langerhans in the pancreas have been demonstrated to affect xenobiotic metabolism in experimental animals (see next section).

1.5.1 Liver, Cytochrome P-450 and Diabetes Mellitus:

The first report suggesting that diabetes mellitus may affect drug metabolism (Dixon *et al.*, 1963) showed an overall decreased hepatic microsomal metabolism following acute treatment with the β-cell toxin, alloxan, and that this effect was suppressed by insulin. It was reported that activities of drug-metabolizing enzymes in liver microsomes, such as hexobarbitone hydroxylation were markedly decreased by alloxan-induced diabetes in male rats. A later study by Kato *et al.*, (1971) demonstrated that the magnitude of the difference spectum of cytochrome P-450 induced by hexobarbital and aminopyrine was markedly decreased in male but not in female alloxan-treated rats. This suggests that the binding capacities of cytochrome P-450 for hexobarbital and aminopyrine, which are normally stimulated by androgen (Schenkman *et al.*, 1990) are decreased in the microsomes of diabetic male rats but not in female rats and this was confirmed by other workers (Kato & Gillette, 1965; Weiner *et al.*, 1972). However, studies have shown that diabetes produces an increase in the hepatic levels of cytochrome *P*-450 2E1, responsible for the increase in the microsomal metabolism of aniline in rats (Favreau & Shenkman 1987) which means the effect of diabetes is not always to decrease drug metabolism as documented by Dixon *et al.*, (1963). There is an increase in the oxidation of aniline which is known to be an effective substrate for cytochrome P-4502E1 (Defeng *et al.*, 1993). Although the microsomal metabolism of androgenic hormones, testosterone and androst-4-ene-3,17-dione, has been studied in several laboratories.(Conney *et al.*, 1973 Einarsson *et al.*, 1974), Reinke *et al.*(1978) were the first to report alterations in hepatic steroid metabolism in streptozotocin-diabetic male rats. The diabetic state resulted in an overall decrease in the rate of microsomal metabolism of androst-4-ene-3,17-dione compared to the control. This was confirmed by Skett *et al.* (1980). Changes in androst-4-ene-3,17-dione metabolism can be reversed by insulin treatment. If insulin action on steroid metabolism is investigated in isolated hepatocytes, it is found that steroid metabolism is increased by insulin

(Hussin & Skett, 1987). Metabolism of androst-4-ene-3,17-dione metabolism can, thus, be used a measure of insulin-like activity.

1.6 *Plants used as herbal treatment:*

1.6.1 *ARTEMISIA JUDAICA* (Family Compositae)

The desert plant *Artemisia judaica* L. is widely used in folk medicine in the Arabian region especially in Libya. It is commonly known by the Arabic name 'Shih' and is a popular folk remedy for the treatment of diabetes mellitus in Libya. It is a genus of aromatic and bitter plants of the family Compositae. About 400 species have been cultivated or used by man. It is a genus with alleviating properties for gastrointestinal colics, diarrhoea and gastrointestinal disorders and it is a popular medicine in use for a long time to treat diabetic symptoms and is sold in many markets in Tripoli, Libya. Other uses for this plant have also been reported (Khafagy & Tosson, 1968). It has a protective effect against carbon tetrachloride-induced hepatotoxicity (Kiso *et al.*, 1984). Judaicin and cirsimaritin were isolated from *Artemisia judaica* (Abdalla & Zarga, 1987, Khafagy & Tosson, 1968). *Artemisia* sp. is also known to be used by local populations as an anthelminthic (Khafagy *et al.*, 1971). It has been found to have anti-arteriosclerosis & anti-thrombosis effects (Kaji *et al.*, 1990). Judaicin is a new sesquiterpene ketolactone, recently found to have a cardiotonic effect which did not deviate from the general frame of digitoxin action (Khafagy *et al.*, 1976).

Some of the biological effects of cirsimaritin have been studied besides its antiviral and antibacterial activities, cirsimaritin has been shown to inhibit beef heart cyclic AMP phosphodiesterase, but no information is available on the biological effects of cirsimaritin at the tissue level (Abdalla, & Zarga, 1987).

1.6.2 *ANVILLEA CARCINI* (Family. Compositae)

Anvillea carcinii is a woody desert plant with the aspect of *Astericus graveolens*. It contain hydroxyparthenelide, astriseinolide, kaempferol and quericitine (Elgadi, 1989). but no scientific work has been done on the pharmacology of this plant.

1.6.3 *MARRUBIUM VULGARE* L (Family. Labiatae)

The genus *Marrubium* is represented by 97 species which are widely spread over the temperate and warm regions. Two species have been identified namely, *Marrubium alysson* and *Marrubium vulgar*. Many *Marrubium* species are reported in the literature to be used in folk medicine (Watt, 1962). It is used in the form of a decoction as a remedy for diabetes, anaemia and as a diuretic. *Marrubium* sp. contain marrubiin, tannin, resin, volatile oil, and vitamins (Elgadi, 1990). In north Africa, the tops are used as a flavouring agent (Lewis, 1977).

1.7 Experimental Models of Diabetes mellitus:

Insulin-dependent diabetes is a pathological state that affects endogenous metabolism and also the pathways of metabolism and disposition of drugs as discussed above. Chemical compounds that specifically damage the pancreatic islet β-cells constitute a class of diabetogenic agents. Among such compounds, alloxan and streptozotocin exhibit this characteristic and are widely used to induce experimental diabetes, which closely resembles insulin-dependent diabetes mellitus (Agrawal, 1995). The earlier extensive use of alloxan has more recently been replaced by the streptozotocin-induced diabetes model because streptozotocin is a more selective β-cell toxin (Hoftiezer and Carpenter, 1973) and because its effect on the liver has been shown to be due to its diabetogenic action since treatment with insulin could reverse the effects seen (Favreau and Schenkman, 1987). On the other hand, alloxan has been criticized for its overt toxicity (Hoftiezer and Carpenter 1973) which indicates that this may not be a good model of diabetes. The dose of STZ which was used was 65mg/kg because it has previously been shown in this laboratory to induce hyperglycaemia and hyperlipidaemia in adult rats (Skett, 1986).

Selecting an appropriate model for testing the anti-hyperglycaemic effects of drugs is complicated by the heterogeneity of the diabetic condition and the fact that no

single model is entirely representative of a particular type of human diabetes (Bailey & Flatt, 1990).

Diabetes mellitus could be induced in experimental animals by administering to the animals streptozotocin. STZ is transported into pancreatic beta-cells through glucose transporters in the cell membranes and attacks mitochondria. Mitochondrial ATP generation is inhibited and the resulting high concentration of intracellular ADP causes its degradation providing hypoxanthine, a substrate of xanthine oxidase (XOD) whose activity is intrinsically very high in the beta-cell. The XOD-catalyzed reaction produces uric acid and oxygen radicals, but beta-cells are inefficient in scavenging these radicals because of their extremely low activity of superoxide dismutase. On the other hand, STZ directly activates XOD and enhances oxygen radical generation. Consequently, the pancreatic B-cells are subjected to a high concentration of oxygen radicals when exposed to STZ (Kawad, 1992) and, thus, leading to cellular toxicity. The STZ-diabetic rat model was, thus chosen to represent type1 diabetes mellitus.

1.8 Aims of the Project:

1- In 1988, Hussin *et al.*, showed the role of insulin in the regulation of hepatic steroid and drug metabolism. It was found that addition of insulin to cultures of adult rat hepatocytes caused a general increase in the steroid metabolizing activity of the cells. We wished to use this as a test system for the possible insulin-like effects of extracts of *Artemisia judaica, Marrubium vulgar, & Anvillea carcinii.* We also wished to examine if extracts of *Artemisia judaica, Marrubium, & Anvillea* can enhance insulin action on isolated liver cells. If we could demonstrate such effects we would continue by trying to purify the active ingredients from the extracts.

2- To examine the effects of *Artemisia judaica, Anvillea carcinii L.* and *Marrubium vulgare* on some hepatic functions that are responsible for regulating glucose output. In particular, we wish to examine the ability of *Artemisia judaica, Anvillea carcinii L.* and *Marrubium vulgare* to mimic insulin and attenuate glucagon action on the liver by measuring the activity of glycogen phosphorylase *a* in normal and diabetic rat hepatocytes.

3- To test the effects of *Artemisia judaica* extract in normal and diabetic rats *in-vivo* by giving it in their drinking water on diabetic related parameters i.e. body weight, food intake, water intake, urine production, faeces production and urinary glucose concentration.

4- To investigate the effects of *Artemisia judaica* on internal parameters (e.g liver weight, liver protein content, enzymatic activities of cytochrome P-450 and liver microsomal metabolism of androst-4-ene-3,17-dione.

5- To investigate the effects of *Artemisia judaica* extract on blood glucose in non-insulin-dependent and insulin-dependent diabetic patients, and any potential side effects.

METHODS AND MATERIALS

2. MATERIALS AND METHODS

2.1 Preparation of Artemisia judaica *extract*

Artemisia judaica was collected in the spring from the Huon desert region of Libya and air-dried before transport to Glasgow. A voucher specimen of the plant was identified by Dr. J.H. Dickson, Department of Botany, University of Glasgow. The dried whole plant was kept in the dark in dry conditions until extracted. *Marrubium vulgare* and *Anvillea carcinii* were bought from markets in Libya and a voucher specimen similarly identified by Dr. Dickson.

2.1.1 Preparation of crude extract

The whole plant (aerial parts) was crushed in a grinding machine to a fine powder and was kept in nylon bags at room temperature until used. The powder (100g) was mixed with 500ml of distilled water and allowed to extract overnight at room temperature. The resultant dark green-brown mixture was filtered through filter paper (Whatman No.1) and the filtrate was evaporated to dryness at 40°C under reduced pressure in a Buchi Rotavapor-R rotary evaporator. The resultant brown powder weighed 16g. The residue was redissolved in distilled water at a concentration of 0.2g/ml and kept frozen in 1ml aliquots at -20°C until required (see Figure 2.1)

This was termed the crude extract. The same method was used for the preparation of the crude extracts of *Marrubium vulgare* and *Anvillea carcinii.*

2.1.2 Preparation of the aqueous and ethanolic extract of *Artemisia judaica*

The dried crude extract (14g), produced as above, was mixed with absolute ethanol (80 ml) and allowed to stand overnight in a dark place. The resultant light green-brown mixture was then filtered and the filtrate was evaporated to dryness at 40°C under reduced pressure. 2g of dry material was found and redissloved in absolute

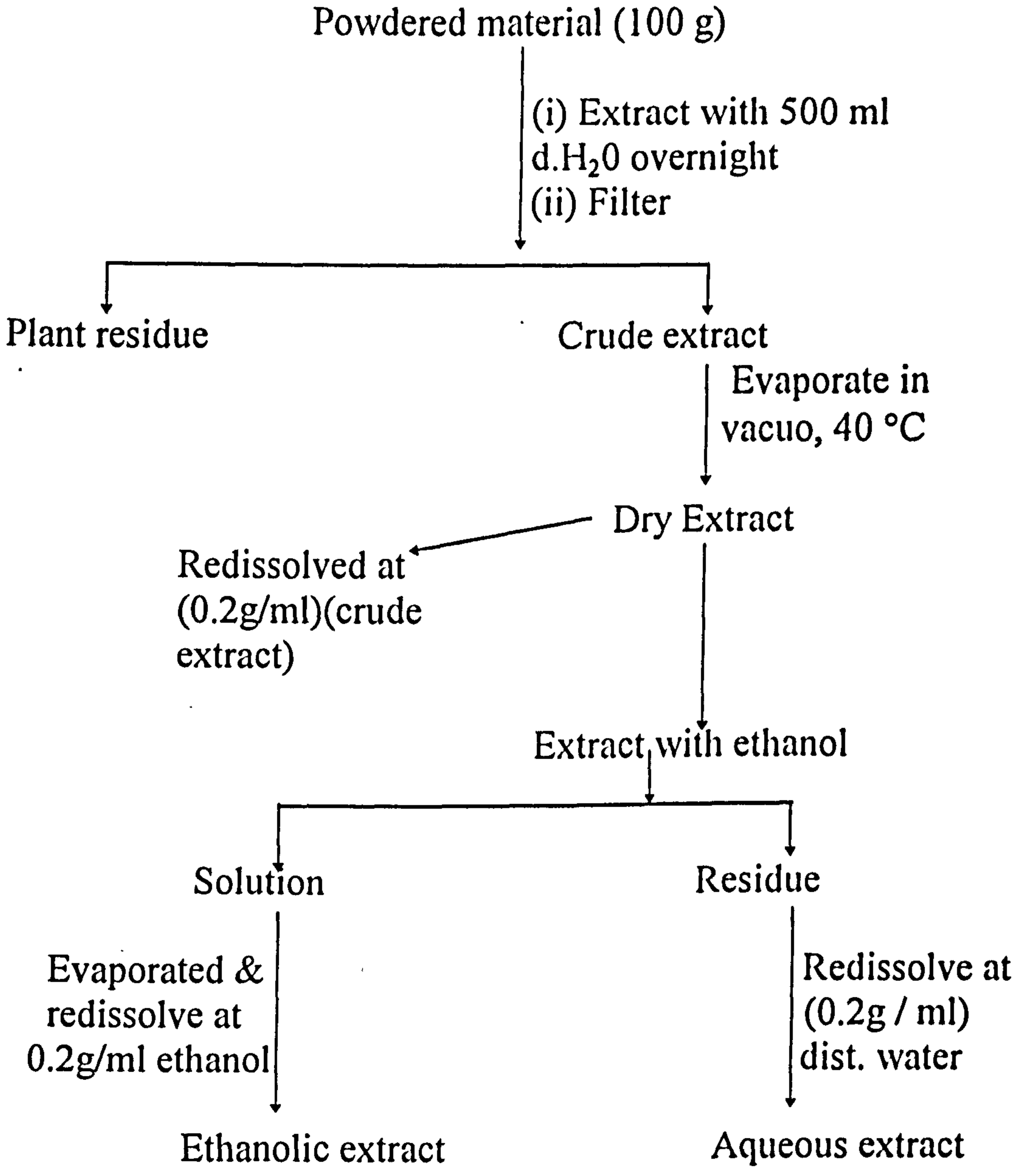


Figure 2.1 Extraction procedure for *Artemisia judaica* (Crude, Aqueous and ethanolic)

ethanol (0.2g/ml). This was termed the "ethanolic extract". The residue from the extraction was redissolved in distilled water (0.2g/ml) (Figure 2.1).

2.2 *IN-VIVO* STUDIES

2.2.1 EXPERIMENTAL ANIMALS

Mature male Wistar rats of approximately 250g were used throughout the study. The animals were bred in the department and housed in light (lights on 0800-2000hrs) and temperature (19°C ± 1°C) controlled conditions. The animals had free access to food (CRM Nuts, Labsure, Croyden) and tap water throughout the experiments except where noted.

2.2.2 Induction of diabetes mellitus

Diabetes mellitus could be induced in experimental animals by administering to the animals streptozotocin (STZ). It is considered that STZ may provide a better model for type 1 (insulin-dependent) diabetes than alloxan because of its more selective beta-cell toxicity (Hoftiezer & Carpenter, 1973). Since STZ is unstable in solution, it was dissolved in distilled water (at 65mg/ml.) immediately before use and then injected intravenously (65mg/kg) into the tail vein of the rat under halothane/nitrous oxide anaesthesia. The animals were replaced in ordinary cages and left for three days for recovery and development of the symptoms of diabetes mellitus before use.

2.2.3 METABOLIC CAGES

The metabolism cages were supplied by Jencons of Hemel Hempstead and were of glass construction with wire mesh floor and roof. The cages were set-up in the animal house to keep the animals in the same light and temperature conditions that they were previously accustomed to. The cage design allows the animals easy access to measured quantities of food and fluid with a facility to collect any crumbs or drips to allow

accurate measurement of the animals' consumption. The wire floor allows faeces to fall throughout the living area to facilitate collection. Urine either falls or is deflected onto the walls of the chamber below the living area, and runs down the glass to be collected in a flask. Each cage was initially provided with 100g of food and 100mls of water which were refreshed and replenished as required.

2.2.4 EXPERIMENTAL PROCEDURES

Normal, healthy male rats or rats made diabetic by treatment with STZ as described above were used in experiments lasting up to 27 days in total. At no time were animals in the metabolic cages for more than 7 days continuously. The actual experimental protocols are outlined in Figures 2.2 - 2.4. Each animal acted as its own control being tested as control, after induction of diabetes (if appropriate) and after treatment with the respective extracts.

2.2.5 Experimental protocol:

Every 24 hours the following parameters were measured:

1. Body weight
2. Food intake
3. Fluid intake
4. Urine volume
5. Faeces weight

A urine sample was collected and frozen until assayed for glucose and ketones as described later.

The animals were removed from their cages for a period not exceeding 5 minutes to facilitate collection and measurement of the required parameters. Although the animals did urinate and defaecate during this period the quantities involved were not significant compared to the daily collection.

2.2.6 Normal rat treated with *Artemisia* (17 Day experimental run):

Animals were weighed (starting weight: 250-300g) and placed in the metabolic cages. The animals were allowed a 24hr run-in period to adjust to their new surroundings after which the experimental run began. Each animal was then tested under control conditions for 7 days. The animal was then removed from the metabolic cage and allowed to recover for 3 days in an ordinary animal cage.

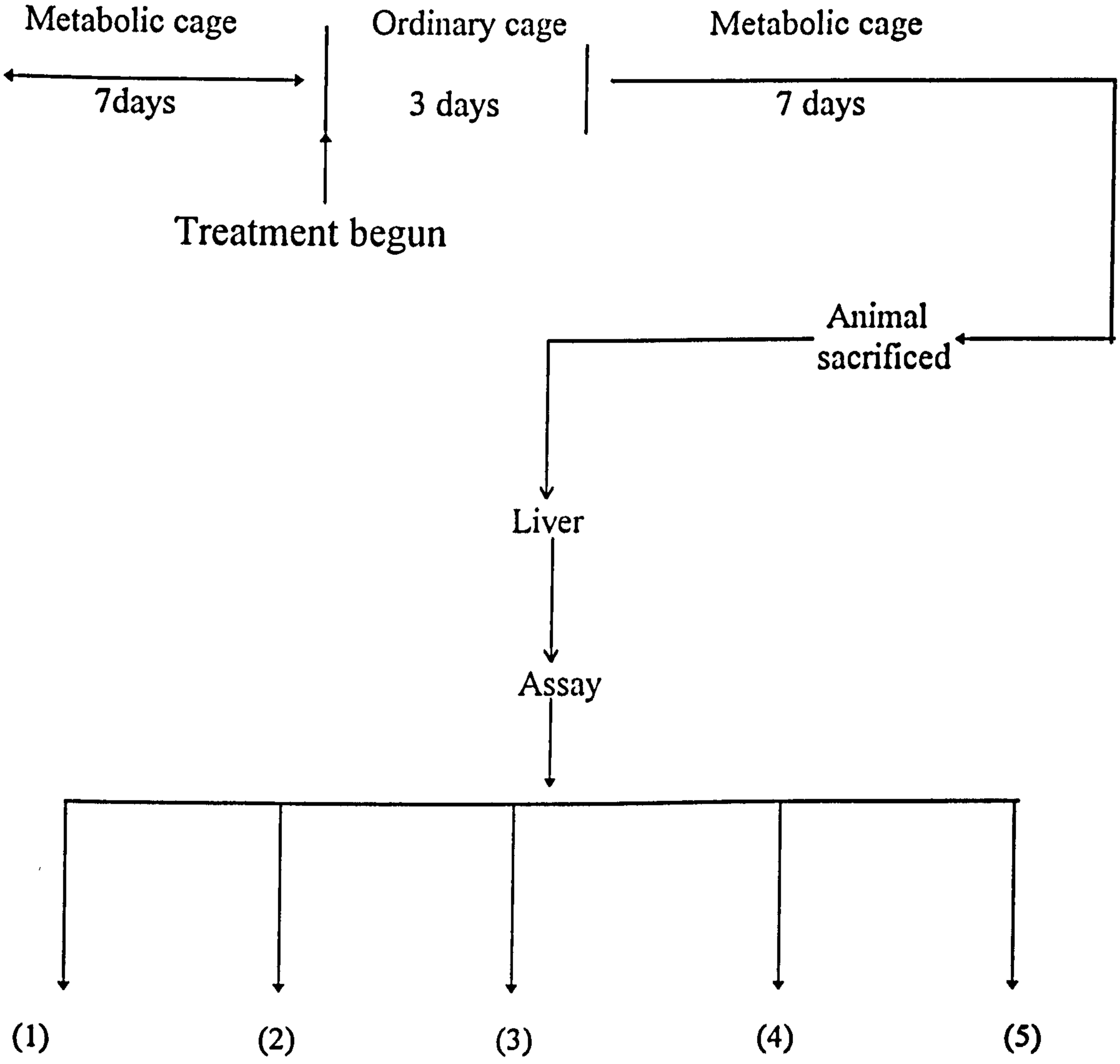
On day 11 (for animals 1-3) or day 7 (for animals 4-9) animals were supplied with the plant extract as a decoction in their drinking water - 5ml of *Artemisia* crude extract as prepared above in 200ml tap water (Figure 2.2).

2.2.7 Diabetic rat (17 Day experimental run):

The animals were selected and run as controls in the metabolic cages for 7 days to ensure they exhibited a normal pattern of measured parameters. Diabetes mellitus was induced on day 7 by administration of STZ as described in Section 2.2.2, after which the animals were kept for three days in a conventional cage to allow recovery and onset of diabetes. On day 11 the animals were replaced in the metabolic cages and the experiment continued for a further 7 days (Figure 2.3).

2.2.8 Diabetic rats treated with *Artemisia* extract (27 day experimental run):

The animals were selected and run as controls in the metabolic cages for 7 days to ensure they exhibited a normal pattern of measured parameters. Diabetes mellitus was induced on day 8 by administration of STZ as described above, after which the animals were kept for three days in a conventional cage to allow recovery and onset of diabetes. On day 11 the animals were replaced in the metabolic cages and the experiment continued for a further 7 days. The animals were removed from the metabolic cage and



(1) = Protein content

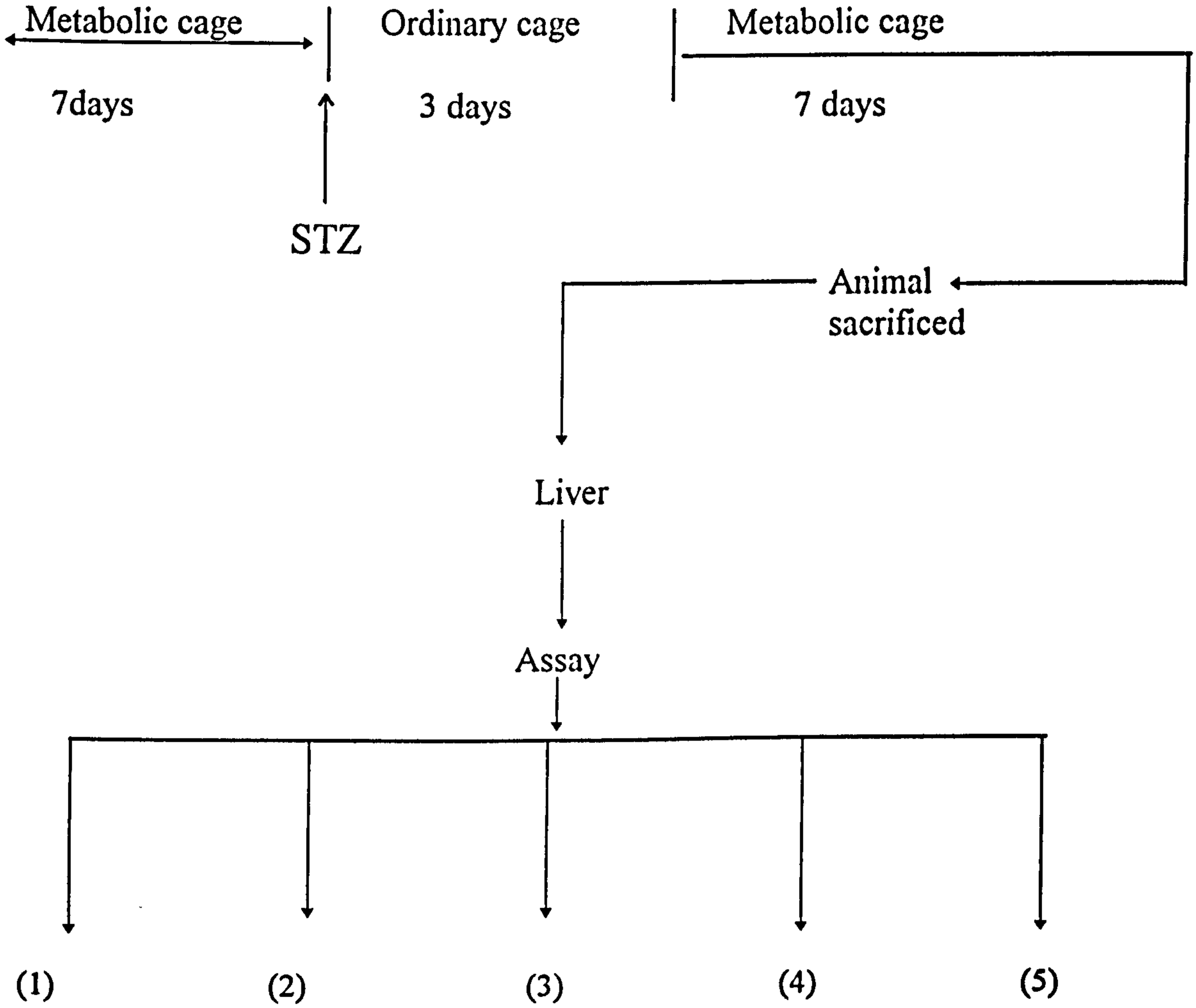
(2) = Cytochrome P-450 content

(3) = Aniline 4-hydroxylase metabolism

(4) = Aminopyrine N-demethylase metabolism

(5) = Androst-4-ene-3,17-dione metabolism

Figure 2.2 Normal rat treated with *Artemisia* extract (10 days).



(1) = Protein content

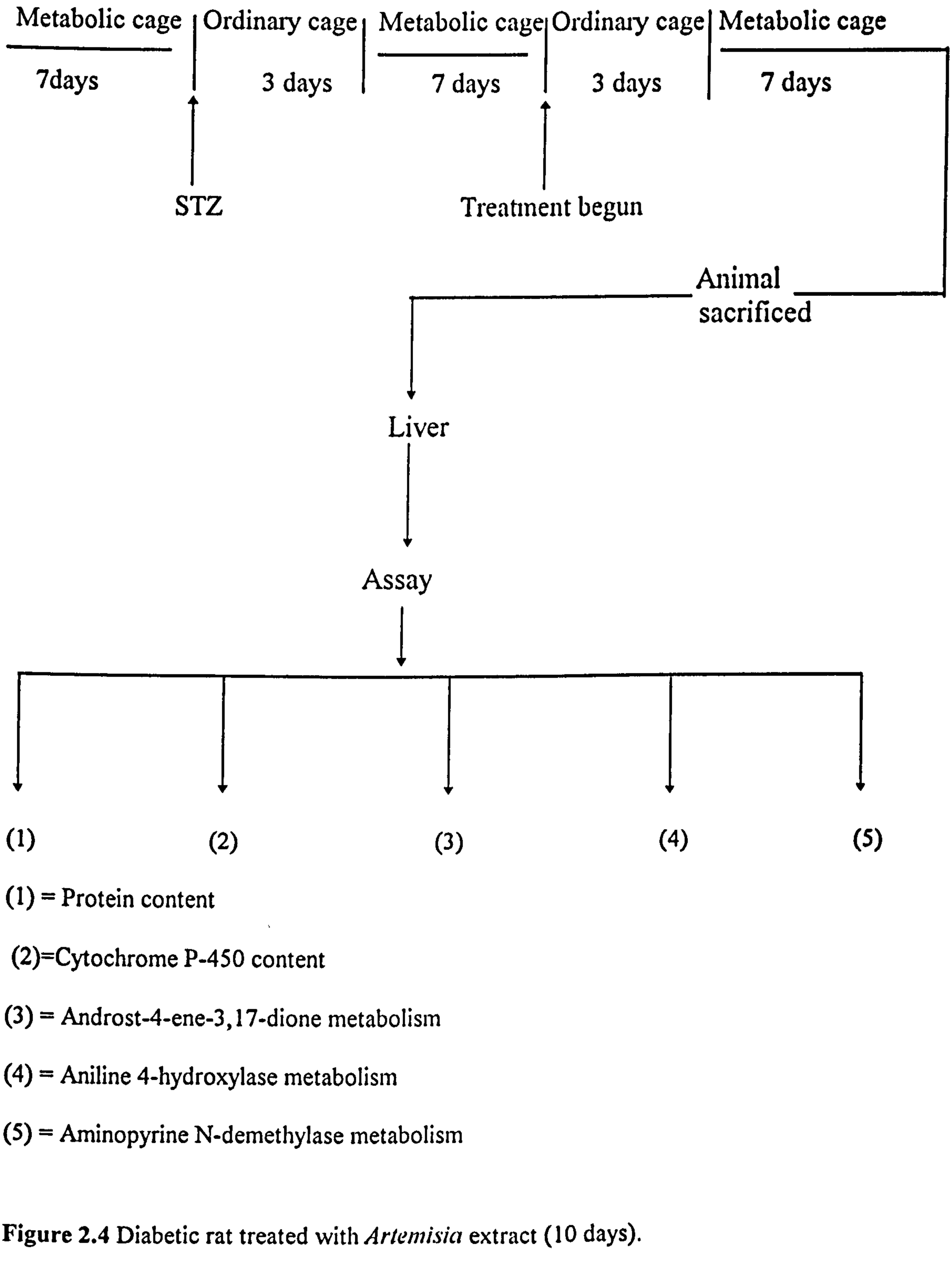
(2)=Cytochrome P-450 content

(3) = Aniline 4-hydroxylase metabolism

(4) = Aminopyrine N-demethylase metabolism

(5) = Androst-4-ene-3,17-dione metabolism

Figure 2.3 Diabetic rat without treatment (10 days).



(1) = Protein content

(2)=Cytochrome P-450 content

(3) = Androst-4-ene-3,17-dione metabolism

(4) = Aniline 4-hydroxylase metabolism

(5) = Aminopyrine N-demethylase metabolism

Figure 2.4 Diabetic rat treated with *Artemisia* extract (10 days).

kept in a conventional cage for 3 days during which time they were treated with *Artemisia* extract (concentration as given above). The bitter taste of *Artemisia* extract meant that the animals did not drink it, so, in these cases, a more dilute solution was used for day 19, (1 or 2ml of *Artemisia* extract in 200ml of tap water) followed by a more concentrated solution on day 20 (2.5 or 3ml of *Artemisia* extract in 200ml tap water) and then to the normal concentration of extract on day 21 and on day 21 the animals were returned to the metabolic cage for a final period of 7 days (Figure 2.4).

2.3 Assays

2.3.1 Glucose Assay

Urine samples were assayed for glucose using the assay kit supplied by the Sigma Chemical Co. Ltd. which is based on a procedure similar to that described by Carrol, *et al.*, (1970) based on the following reactions:

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{Hexokinase}} \text{G-6-P} + \text{ADP}$$

$$\text{G-6-P} + \text{NADP}^{+} \xrightarrow{\text{G-6-PDH}} \text{6-PG} + \text{NADPH}$$

$$\text{NADPH} + \text{PMS} \rightarrow \text{NADP}^{+} + \text{PMSH}$$

$$\text{PMSH} + \text{INT} \rightarrow \text{PMS} + \text{INTH}$$

The analysis is based on the hexokinase (HK) catalyzed conversion of glucose to glucose-6-phosphate (G-6-P). This reaction is coupled to the subsequent reduction of

nicotinamide adenine dinucleotide phosphate (NADP) to NADPH by the action of glucose-6-phosphate dehydrogenase (G-6-PDH). In the presence of NADPH, phenazine methosulphate (PMS) is reduced. The PMSH generated is then responsible for the reduction of iodonitrotetrazolium chloride (INT) forming INTH, which is measured colourimetrically at 520nm. The colourimetric response is proportional to the glucose concentration. The absorbance of the sample was thus measured at 520nm in a Shimadazu UV-240 spectrophotometer and the glucose concentration calculated from standards supplied by Sigma

2.3.2 Urine ketones and glucose

The presence of ketones in the urine was assayed using Keto-Diabur 5000 strips supplied by Boehringer, Mannheim, Germany which produce a colour change in the presence of ketones.

2.4 MEASUREMENT OF OTHER PARAMETERS

After the various treatments, described above, the animals were sacrificed by carbon dioxide asphyxiation, and their necks were broken. Immediately after cervical dislocation, the throat was cut and blood was collected by using a 10ml syringe. 5ml of blood was transferred to a lithium heparin bottle containing 100µl of Trasylol. The livers were quickly removed from the animal as soon as possible, rinsed in ice cold phosphate buffer (pH 7.4, 0.01 M with 1.15% potassium chloride added) to remove any excess blood and other foreign material , blotted dry on filter paper and weighed. At the end of each experiment, therefore, there was urine, blood and liver material to be assayed. The urine was assayed for glucose and ketones (see section 2.3.2), the blood for insulin and glucagon and the liver was assayed for protein content, cytochrome P-450 content,

activity and the metabolism of androst-4-ene-3,17-dione, aniline and aminopyrine. All of these assays are described below.

2.4.1 Preparation of liver microsomes

In order to measure the liver parameters, subcellular fractions needed to be prepared. It is essential to keep the liver as cool as possible and to get the microsomes prepared and frozen as quickly as possible to prevent damage to the tissue and its enzymes.

The liver was homogenized in five times its weight of phosphate buffer containing 1.15% potassium chloride (see section 2.20.1), in a Potter-Elvehjem homogenizer with a Teflon pestle (for 1 g of liver use 5ml of buffer). The homogenate was then centrifuged in a Sorvall refrigerated centrifuge for 15 minutes at 12500xg to sediment the mitochondria and nuclei. The post-mitochondrial supernatant then had calcium chloride (1M) added to it to bring the supernatant to 8mM with respect to calcium chloride (i.e. 0.1 ml of 1M calcium chloride (BDH Chemicals) for every 12.5 ml of supernatant). The solution was then mixed and left to stand on ice for 5 minutes. The supernatant was then centrifuged again this time at 28000xg for 20 minutes to sediment out the microsomes. The supernatant was poured off and the pellet resuspended in the phosphate buffer. For every 50 volumes of the original homogenate, the sediment was resuspended in 20ml buffer and 5ml glycerol. The microsomes were then frozen at about -70°C. The microsomes were used to measure the hepatic parameters discussed above and described in detail below. All assays have previously been tested for linearity with respect to time and concentration of measured compound in the range used in this study. Enzyme assays have also been previously tested to be within the linear range for protein concentration.

2.4.2 Protein determination

The method described by Lowry et al (1951) was used to determine the protein content of the microsomes. A sample of the prepared microsomes was diluted (1:100) with distilled water. A reagent was made by mixing 50 parts of 4% (weight / volume) sodium carbonate dissolved in 0.1 N sodium hydroxide (Fisons), 1 part 1% (weight / volume) potassium tartrate (BDH Chemicals) and 1 part 0.5% (weight / volume) $CuSO_4.H_2O$ (Glasgow University Chemistry Department). This reagent must be freshly made as it only lasts 3-4 hours. 2ml of this reagent was then added to 1ml of diluted microsomes, mixed and left to stand for 10 minutes. Then 0.3 ml of 1N Folin Ciocalteu reagent (BDH Chemicals) was added, mixed and allowed to stand for 30 minutes at room temperature. A coloured complex was formed between the alkaline copper-phenol reagent and tyrosine and tryptophan residue of the protein. The optical density at 750nm was determined on a Shimadzu UV-240 spectrophotometer. A standard curve was also prepared using 0.2 to 1ml aliquots of crystalline bovine serum albumin (200µg/ml, Sigma BSA fraction IV) made up to 1ml with distilled water. By comparing the optical density of the sample to the standard curve, a measure of the protein content was established.

2.4.3 CYTOCHROME P-450 ENZYME ACTIVITIES

2.4.3.1 ASSAY OF ANILINE 4-HYDROXYLASE ACTIVITY

This assay acts as a measure predominantly of the enzyme activity of cytochrome P4502E1, (aniline is hydroxylated to 4-aminophenol) as follows (Gibson & Skett, 1994) (Figure 2.5).

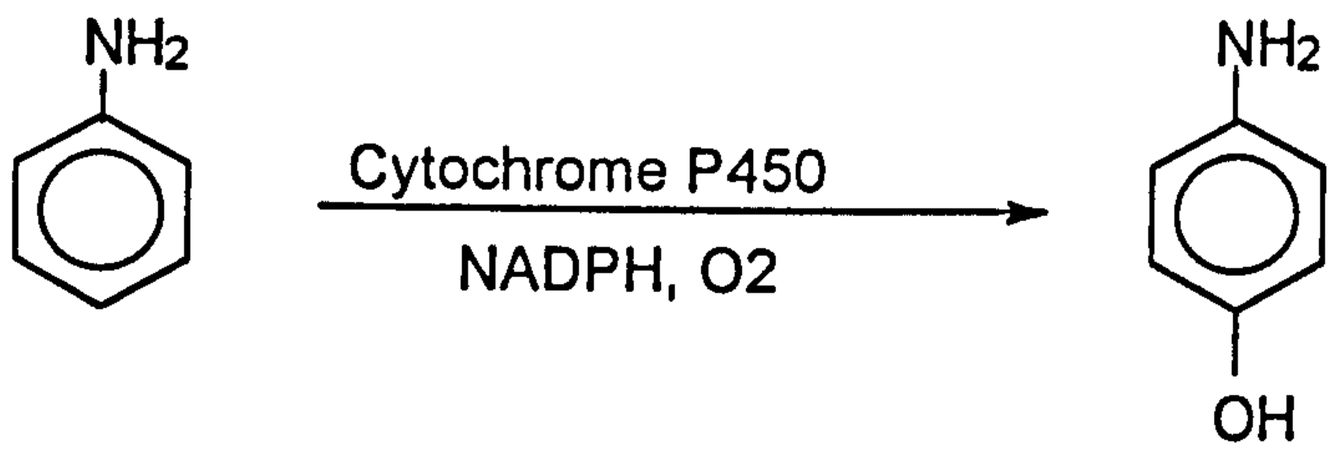


Figure 2.5 The hydroxylation of aniline

The following was added to each test tube :

Microsome preparation 1ml

0.1M Magnesium Chloride (BDH Chemicals) 0.1ml

0.5M Nicotinamide (Sigma Chemical Co.Ltd.) 0.1ml

Tris buffer pH 7.4 0.05M 0.59ml

0.05M Aniline (BDH Chemicals) 0.1ml

Co-factor mixture 0.11ml

(to start reaction. see reagent section 2.20 at the end)

The above were incubated at 37°C for 30 minutes in a shaking water bath before the reaction was stopped by adding 1ml of 20% trichloroacetic acid (BDH Chemicals), which precipitated the protein present in each incubation. This was mixed well and allowed to stand for 5 minutes at 4°C. The precipitate was then spun down in a bench centrifuge for 15 minutes. 1ml of the supernatant was then taken to be assayed for 4-aminophenol. To the 1ml supernatant was added 1ml of 1% phenol in 0.5M sodium hydroxide solution (Fison) and mixed with 1ml of sodium carbonate (4% w/v) solution (Hopkin and Williams). The solution was then mixed and the resultant solution was left to stand for 30 minutes at room temperature before the optical density was measured at 630nm in a Shimadzu UV240 spectrophotometer. The measurement obtained was compared to the standard curve to find the concentration of 4-aminophenol formed per minute per mg protein (see calculations in Section 2.11)

The standard curve was prepared by using 0.2 to 1ml aliquots of fresh 4-aminophenol solution (10μM) (BDH Chemicals) made up to 1ml with distilled water.

Again the optical density was measured at 630nm and a standard curve was drawn from the values obtained.

2.4.3.2 ASSAY OF AMINOPYRINE *N*-DEMETHYLASE ACTIVITY

This assay is considered to act as a general measure of the enzyme activity of cytochromes P450 of the 2B and 2C subfamilies.

N-demethylation of drugs is a common metabolic pathway and is thought to proceed by initial hydroxylation at the alpha-carbon atom and subsequent breakdown of the carbinolamine intermediate liberating formaldehyde (Gibson & Skett, 1994) (Figure 2.6).

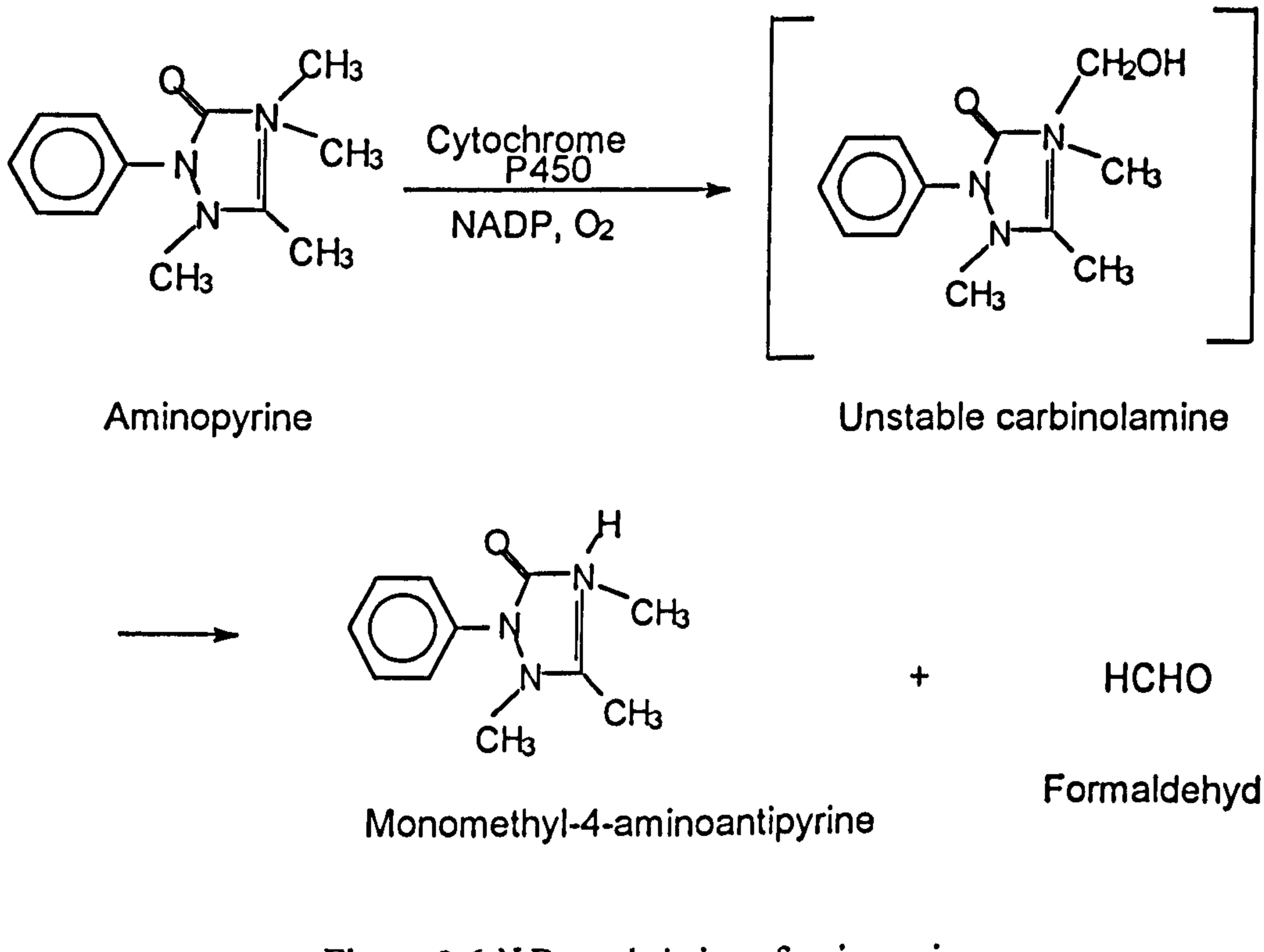


Figure 2.6 *N*-Demethylation of aminopyrine.

The following were added to each test tube :

Microsome preparation 1.00ml

0.1M Magnesium chloride (BDH Chemicals) 0.10ml

0.5M Nicotinamide (Sigma Chemical Co.Ltd) 0.10ml

450mM Semicarbazide (Sigma Chemical Co.Ltd.) 0.10ml

0.5M phosphate Buffer pH 7.4 ..... 0.49ml

50mM Aminopyrine (Sigma Chemical Co.Ltd.) 0.10ml

Co-factor Mixture 0.11ml

(to start the reaction, see reagent section 2.20 at the end)

The above mixture was incubated at 37°C for 30 minutes in a shaking water bath before the reaction was stopped with 1ml 20% zinc sulphate (Hopkin and Williams), which precipitates the protein present in each incubation. This was then mixed well and left for 5 minutes. 1ml of saturated barium hydroxide (Hopkin and Williams) solution was then added to precipitate out the zinc sulfate. Again this was mixed and left to stand for 5 minutes. The precipitate was then spun down in a bench centrifuge for 15 minutes. 2ml of the supernatant was added to 1ml of Nash reagent (see reagent section 2.20.2) for the assay of formaldehyde. The supernatant / Nash reagent mixture was incubated for 30 minutes at 60°C in a water bath, using a glass marble to close the mouth of tube to cut down on evaporation. The optical density was measured at 415nm against distilled water as a blank. The measurement obtained was compared to the standard curve obtained using a standard formaldehyde solution to find the concentration of formaldehyde formed per minute per mg protein (see calculations in section 2.11)

2.4.3.3 ASSAY OF STEROID (ANDROST-4-ENE-3,17-DIONE) METABOLISM

The activity of several enzymes upon the substrate androst-4-ene-3,17-dione was studied using a technique outlined by Berg and Gustafsson in 1973. These enzymes being 7α-, 16α- and 6β-hydroxylases, 17-oxosteroid oxidoreductase and 5α-reductase. They act on the steroid substrate to produce the various products shown in (Figure 2.8 and 2.9). A co-factor mixture was prepared in the same way as that for the assay of

aniline 4-hydroxylase activity. It was preincubated at 37°C for 2 minutes to allow the formation of NADPH. 1.9ml of phosphate buffer pH 7.4, 1ml microsomes, 500µg of [4-^{14}C]-androst-4-ene-3,17-dione (containing 0.1µCi, dissolved in 50µl of acetone) were then added and incubated in a shaking water bath at 37°C for 10 minutes. The metabolites formed were extracted and quantitated as described later (see Section 2.16).

2.5 HUMAN STUDIES

2.5.1 Preparation of *Artemisia judaica* extract

500g of *Artemisia judaica* containing stems and leaflets were added to 2 litres of tap water and were boiled for a period of 30min. After cooling to room temperature the extract was sieved to remove stems and leaflets and stored in a refrigerator (4°C). Each patient drank a half glass (approx. 100ml) each eight hours.

2.5.2 Subject Descriptions

Patient (1): was a man of 43 years with mild diabetes of 5 years duration. This patient had undergone therapy with *Artemisia judaica* and *Marrubium vulgare* with a controlled diet for approximately three years. The patient was asked to stop the treatment with *Artemisia judaica* and *Marrubium vulgare* extract for the period of the study.

Patient (2): was a man of 20 years who had been treated with insulin injection (40 i.u. once daily). The patient was then given *Artemisia judaica* extract as described three times daily together with the pre-existing insulin therapy.

Patient (3): was a man of 17 years. Since being a juvenile he had been treated with plants extracts one of them being *Artemisia judaica*. He had stopped using the *Artemisia* because when he took it he suffered from bradycardia. He had asked the

doctor to replace his treatment with lente insulin injection. We asked the patient to use *Artemisia judaica* with insulin. The patient had polyuria, polydipsia and polyphagia with insulin treatment.

Patient (4): was a man aged 24 years with diabetes of one year duration, he was treated with diet restriction and insulin (30 i.u. a.m. and 20 i.u. p.m.). This was the first time he had used *Artemisia* extract but he knew that the plant is widely used by diabetics.

Patient (5): was a woman of 55 years with diabetes mellitus of eight years duration, she used hypoglycaemic agents and *Artemisia* and other plants at some times. This patient was asked to stop the use of these plants

Patient (6): was a man of 60 years with mild diabetes of 20 years duration. He had used this plant for a long time but at irregular intervals and he did not feel any side effects. Some history was taken as described in the results section.

2.6 IN-VITRO EXPERIMENTS (ISOLATED HEPATOCYTES):

2.6.1 Preparation of isolated hepatocytes

Hepatocytes were isolated from male rats (weight 250 - 350 g) by the collagenase perfusion method of Seglen (1976). The reservoir, kept at 45 °C by means of a heated water-jacket, was filled with Ca^{2+}-free buffer (see Section 2.21.1) which was oxygenated with a slow flow of pure oxygen through the apparatus. The rat was anaesthetised (4% halothane, 800 ml/min N_2O, 800 ml/min O_2 using a Mini Boyle anaesthetic machine), the abdomen cut open and the portal vein freed of any fat. A thread was inserted under the portal vein and was loosely tied. The cannula (size 20 GAX 32mm length) was inserted into the hepatic portal vein at a position just before the vein branches into the liver and the thread tied tightly around the cannula. The needle was removed from the plastic cannula and the blood was allowed to flow back up the cannula. The inferior vena cava was cut to reduce back pressure on the liver and the

cannula was attached via a silicon rubber tube to a pump providing a continuous flow of warmed buffer (37°C). The thorax was opened and the superior vena cava was cut close to the heart to further reduce back pressure on the liver (Figure 2.7). The Ca^{2+}- free Krebs-Henseleit buffer was allowed to pass through the liver for at least 12 min as necessary for disruption of the tight junctions, which intimately connect the parenchymal cells (500ml at 40ml/min). The Ca^{2+} free buffer was then changed to the Ca^{2+} free buffer (without EGTA 100ml see section 2.21.2) and then Ca^{2+} free buffer without EDTA containing collagenase (120 ml at a concentration of 432 units of collagenase/ml and 4.2mM $CaCl_2$ (see section 2.21.3) and this buffer was allowed to run through for 1 min to wash the first buffer out of the system and then the collagenase buffer was recycled for 12 - 15 min at a rate of 40ml/min. By this method of collagenase perfusion it is possible to convert the whole liver to a suspension of intact cells. At the end of the second perfusion with collagenase the liver should look as if it is falling apart and gentle pressure on the lobes should release the cells. At this stage the perfusion was stopped and the liver removed from the animal. The liver capsule was removed and the cells were stroked out into Ca^{2+}-free Krebs-Henseleit buffer . The suspension of cells was filtered through gauze (Snow flake absorbent gauze) to separate connective tissues and undigested liver. The cells were centrifuged in a Damon-IEC refrigerated centrifuge at a speed of less than 200 rpm (50 g) for 2 min at a temperature of 5°C. The cells were washed 3 times in the same buffer and the upper layer was removed by suction after centrifugation on each occasion. After the last wash the cells were resuspended in washing buffer (see Section 2.21.4) and left on ice for 1h. The number of cells was estimated in a haemocytometer. Viability was estimated by microscopic examination, the cells which are highly refractive are live cells and cells with a viability above 90% were used in the experiments. The preparation was diluted to 10 million live cells / ml before subsequent incubation at 37°C under 95% air and 5% CO_2 in a humidified incubator (Forma Scientific Model 3028) for about 30 min to equilibrate the cells.

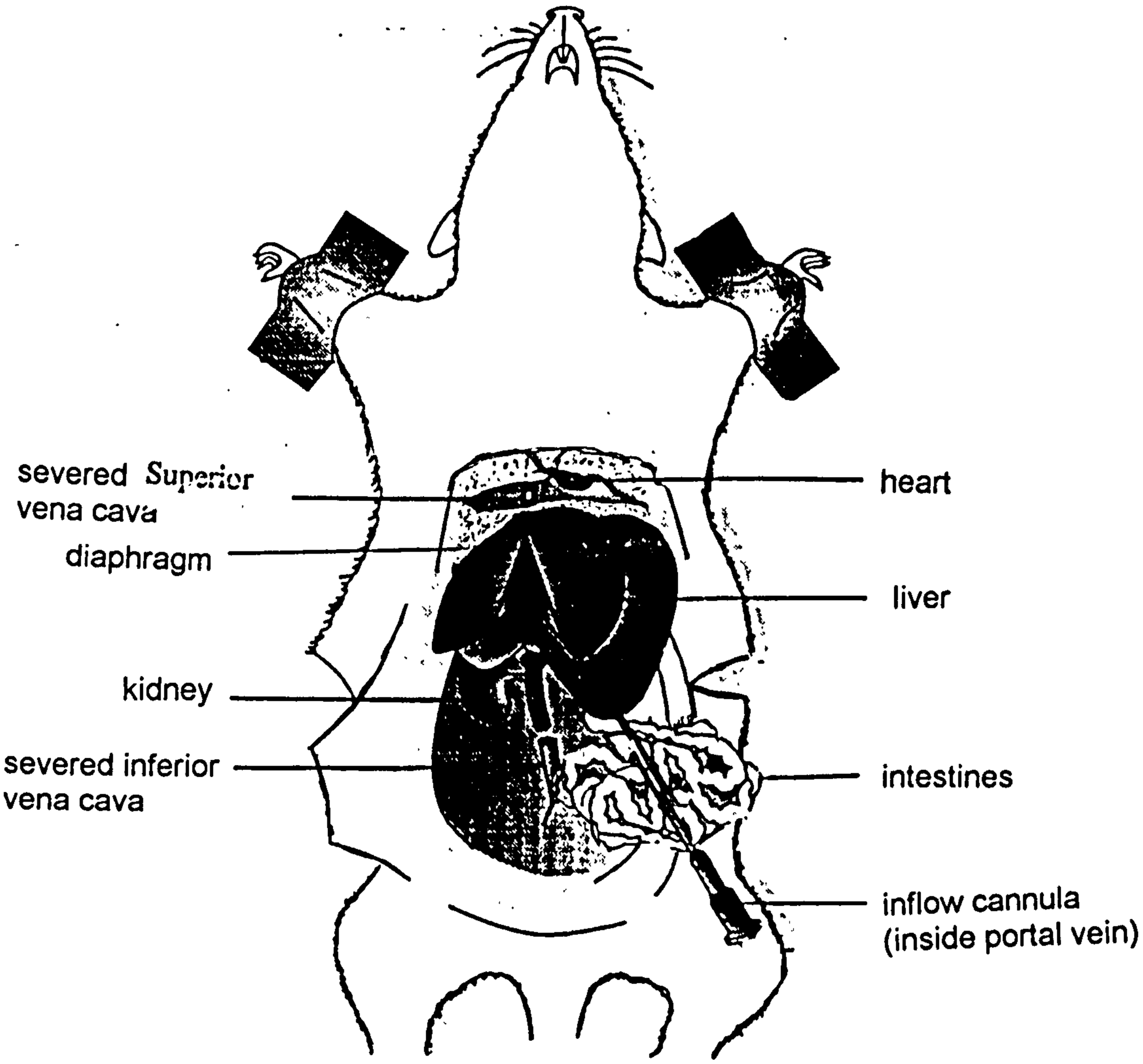


Figure 2.7: Diagram of the liver perfusion (Adapted from Berry *et al.*, 1991).

2.7 Hepatocyte culture

Into separate 9 cm diameter sterile plastic petri-dishes (Nunclon, Denmark) were placed 10^7 cells and 6 ml Ham's F-10 medium containing 0.1% bovine serum albumin plus 0.05 ml of a penicillin / streptomycin mix (100u /100 µg / ml). This procedure was carried out under sterile conditions. The petri-dishes were incubated at 37°C, under 5%CO_2; 95% air for up to 72 hours prior to enzyme assay.

2.8 Addition of test materials

2.8.1 Pre-incubation for androst-4-ene 3,17-dione metabolism assay:

To prepare these cultured cells for assay, the contents of the plates were scraped into separate glass test tubes and centrifuged at a speed of less than 200 rpm (50g) for 2 min. at a temperature of 5°C in a Damon-IEC refrigerated centrifuge. The intact hepatocytes sedimented to the bottom of the tubes and the excess medium was removed using a pasteur pipette. To each tube was added 1 ml of Ham's F-10 or Williams' E medium (containing 0.1% BSA) . The cells are then ready for the addition of the various extracts. If the cells were to be used directly after preparation, they were then suspended in medium following the third wash (see Section 2.6.1 Preparation of Hepatocytes) and used as such.

To 10^7 cells in 1 ml of Ham's F-10 or Williams' E medium as prepared above was added one or more of the following:

1. 10 µl insulin 10^{-7} M dissolved in 0.1M hydrochloric acid (giving final conc. of 10^{-9} M).

2. 10 µl insulin 10^{-4} M dissolved in 0.1M hydrochloric acid (giving final conc. of 10^{-6} M).

3. 10 µl crude extract (as prepared or diluted with solvent to varying degrees.).

4. 10µl of vehicle (0.1M HCl, ethanol or water ; to act as control)

The cells were left in the presence of the test material for 30 min. at 37°C in a humidified incubator in an atmosphere of 5% CO_2 and 95% air. After the preincubation period of 30 min., the cells were centrifuged at a speed of less than 200 rpm (50g) for 2 min at a temperature of 5°C in a Damon-IEC refrigerated centrifuge and the medium was removed using a pasteur pipette.

2.8.1.1 Assay of steroid metabolism

For the assay of steroid metabolism, cells, as prepared above, were resuspended in 1 ml of fresh Ham's F-10 medium or William's E followed by the steroid substrate, 4-[4-^{14}C]-androstene-3,17-dione (100 µg; 0.1 µCi in 17 µl of acetone). The cells were incubated in the CO_2 incubator for 30 min at 37°C. The incubations were terminated by the addition of 0.5 ml of 0.9% NaCl (to increase the ionic strength), 5 ml of Folch solution ($CHCl_3$ (to extract steroid) and MeOH (to precipitate protein); 2:1 v/v) and 500 µg of unlabelled androst-4-ene-3, 17 dione (dissolved in 50µl of acetone; to act as carrier). The tubes were mixed on a rotary mixer and left to stand overnight for phase separation. After 24 hours some tubes did not show phase separation and these were centrifuged at 1000 rpm for 5 min at a temperature of 5°C in a Damon-IEC refrigerated centrifuge for complete separation between the aqueous and organic phases. The lower

organic layer was transferred to another tube and dried at 50°C under oxygen-free nitrogen (to prevent degradation of the metabolites by atmospheric oxygen) using a Techne dry-block. The extract was redissolved in three drops of chloroform using an ultra-sonic water bath for at least 20 seconds and then spotted onto silica gel thin layer chromatography plate (Merck F-254; 20 cm x 10 cm. Individual substrate and metabolite bands were roughly visualized by U.V. light to make sure of adequate separation (Figure 2.9). The accurate bands were located by autoradiography. This step needs complete darkness so the plates were put in a black bag in a closed cupboard for about one week in contact with X-ray film X-Omat film (Kodak, France). The radio-labeled bands were located by developing the film in X-ray developer (DX-24) (200ml added to 1000ml tap water) and then fixing in FX-40 solution (100ml added to 1000ml of tap water) for a few minutes. The film was then washed for 1 hour in cold running water and dried. The developed X-ray film was then aligned with the T.L.C plate (Figure 2.9) and the radioactive bands, which have previously been identified using authentic standards and verified by GC-MS (Gustafsson & Stenberg, 1974), were scraped into polythene scintillation vials and suspended in Ecoscint liquid scintillant. Radioactivity in each sample was measured in a Packard Tri-carb Model 2000 (A) scintillation counter and the various enzyme activities expressed as pmol metabolite formed per min. per mg of protein was determined using the formula for calculations and statistics (section 2.19) from the d.p.m. in each band using a custom-made computer programme.

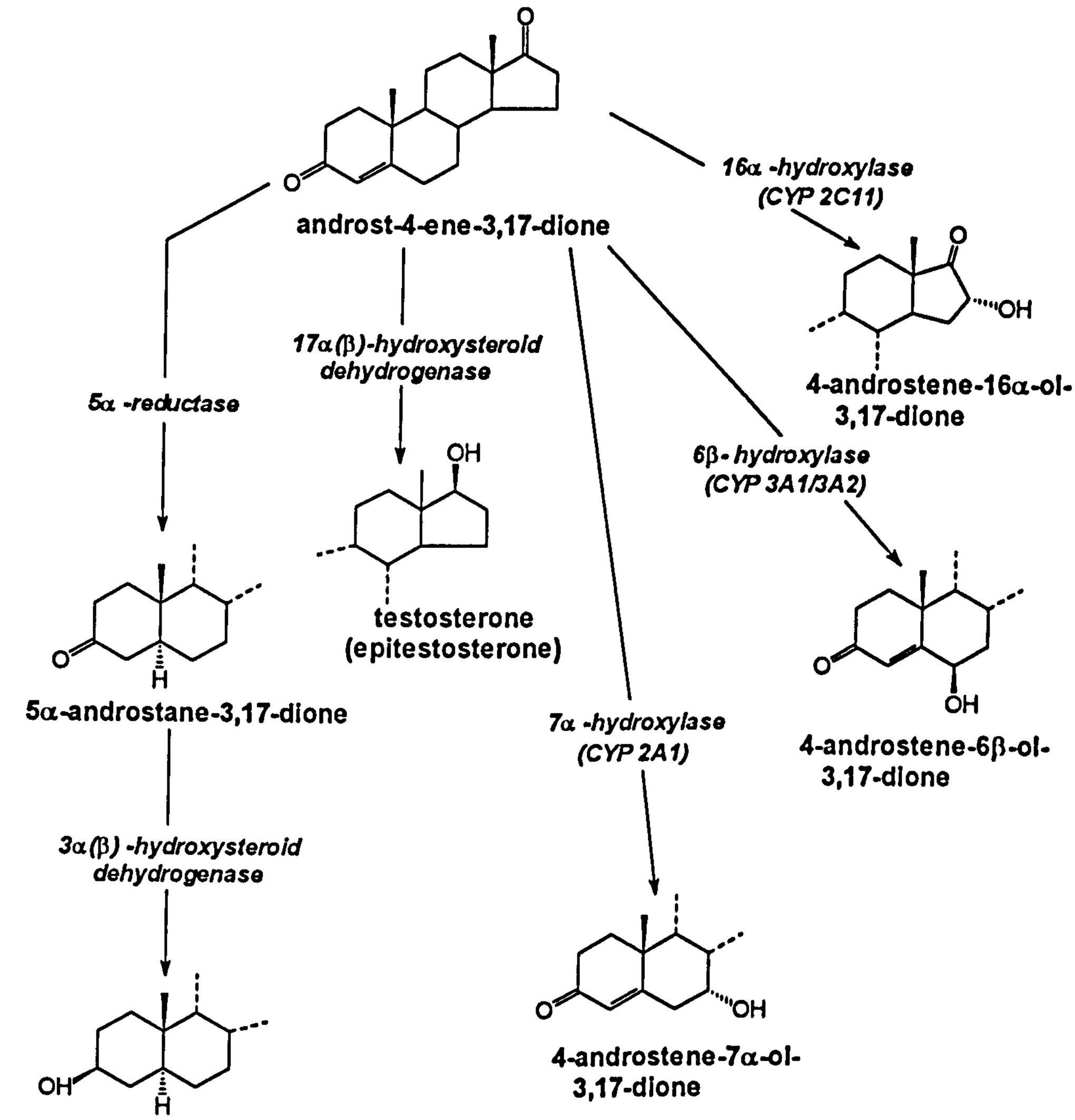
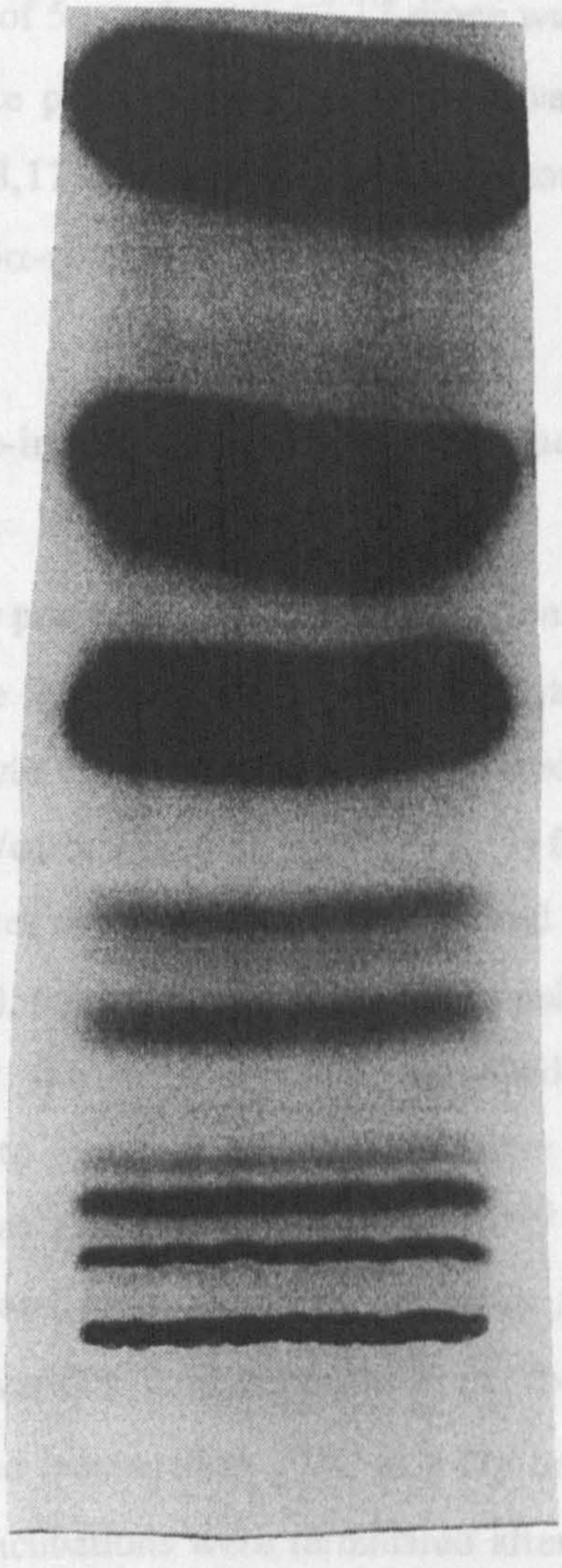


Figure 2.8 The phase 1 metabolism of androst-4-ene-3,17-dione in the liver. Specific isoenzymes thought to produce the various metabolites are given in capital italic.

Figure 2.9 Laser scan of androstene-4-ene-3,17-dione metabolites separation by one-dimensional thin layer chromatography.



5α-Androstane-3,17-dione

Androst-4-ene-3,17-dione (unchanged substrate)

5α-Androstane-3α(β)-ol-17-one

Testosterone/Epitestosterone

Androst-4-ene-16α-ol-3,17-dione

Androst-4-ene-6β-ol-3,17-dione
Androst-4-ene-7α-ol-3,17-dione

Start band

It is apparent from Figure 2.8 that there are six main metabolites of androst-4-ene-3,17-dione which can be separated by this method. However, it has been shown that 3α/β-oxosteroid oxidoreductase does not metabolize the parent compound, but that metabolism by this enzyme occurs subsequent to metabolism by 5α-reductase. Hence, for the purpose of this study, only the activities of the enzymes metabolizing the parent molecule were evaluated and hence the amount of metabolite produced by subsequent metabolism of 5α-androstane-3,17-dione was not determined separately but the amount of metabolite produced by this enzyme was added to the amount of unchanged 5α-androstane-3,17-dione to determine the total amount of metabolite produced by the activity of 5α-reductase.

2.8.2 Pre-incubation for glycogen phosphorylase *a* activity assay.

Cells prepared as outlined in Section 2.6.1 were resuspended in Krebs-Henseleit solution (see Section 2.21.4) at a concentration of 2-3 x 10^6 cells/ml. 2ml aliquots of the hepatocyte suspension were then placed in 20 ml plastic scintillation vials, gassed with oxygen/carbon dioxide mixture (95:5) for approximately 15 seconds and incubated for 30 minutes at 37°C under 95% air and 5% CO_2 in an incubator (Forma Scientific Model 3028), prior to the addition of test substances.

After the pre-incubation described above, 450 µl aliquots of cells were transferred to 1ml microcentrifuge tubes containing 50 µl of drugs at various concentrations (glucagon, insulin at 10^{-10} - 10^{-6} M dissolved in 0.1 M HCl; test compounds and crude extracts, *Artemisia judaica*, *Marrubium vulgare* and *Anvillea carcinii* at various concentrations from 2×10^{-1} - 2 $\times10^{-5}$ g / ml H_2O or vehicle (control)) and incubated at 37°C in a dry heating block (Genesys Instruments) (Figure 2.10). The incubations were terminated after 3 minutes by dropping the tubes in liquid nitrogen (for overall assay procedure see Figure 2.10. The tubes were then stored frozen at -70°C until they were assayed for enzyme activity as described below (section 2.8.2.1). Duplicate incubations were made for each concentration of test substance.

2.8.2.1 Glycogen phosphorylase a assay

Phosphorylase *a* activity was determined by the filter paper assay method of Thomas *et al* (1968) as modified by Stalman and Hers (1975). The method assays glycogen phosphorylase *a* in the 'unphysiological' direction i.e., employing the reaction between D-glucose-1-phosphate (Glc-1-P) and glycogen (i.e. in the direction of glycogen synthesis. The principle involves the incorporation of [^{14}C]glucose from glucose-1-phosphate into glycogen. Caffeine is added to the assay mixture to make the assay specific for phosphorylase *a*. Sodium fluoride and disodium edetate are added to the disruption buffer to inhibit phosphorylase activity.

An equal volume (500µl) of ice-cold disruption buffer (see Section 2.21.5.1) was added to the frozen samples and allowed to thaw on ice and then shaken for about 60 seconds in an ultrasonic bath to disrupt the cells. The mixture was then centrifuged at 2,500*g* at 4°C for 10 min. in a Damon-IEC centrifuge (see Figure 2.10). 50µl of the supernatant was added to 50µl of assay mixture (for composition of assay mixture see below) in a microcentrifuge tube, mixed by means of a rotary mixer and incubated in a water bath at 37°C for 20 mins. The reaction was stopped by removing 30µl aliquots of the mixture and spotting on paper pieces (Whatman 3MM chromatography paper, 10mm^2), previously marked with the sample number in pencil, and dropped in a beaker containing 66% (v/v) ethanol in water. Blank samples were prepared by mixing 50µl of disruption buffer with 50µl of assay mixture.

The filter paper squares were subsequently washed three times for 40 min each in the 66% ethanol on a magnetic stirrer, replacing the ethanol with a fresh mixture after each wash. The stirring bar was screened with an aluminium mesh to prevent disintegration of the filter papers. After the final wash, the ethanol was decanted and the papers washed for 1 minute in acetone. The papers were allowed to dry in air and the radioactivity of each paper measured in a Tri-Carb 2000CA scintillation counter (Packard, Berks., England), by placing the filter papers in 20ml-plastic scintillation vials with 2 ml of Ecoscint scintillation fluid (National Diagnostics, Manville, NJ, USA). (for method flowchart see Figure 2.10)

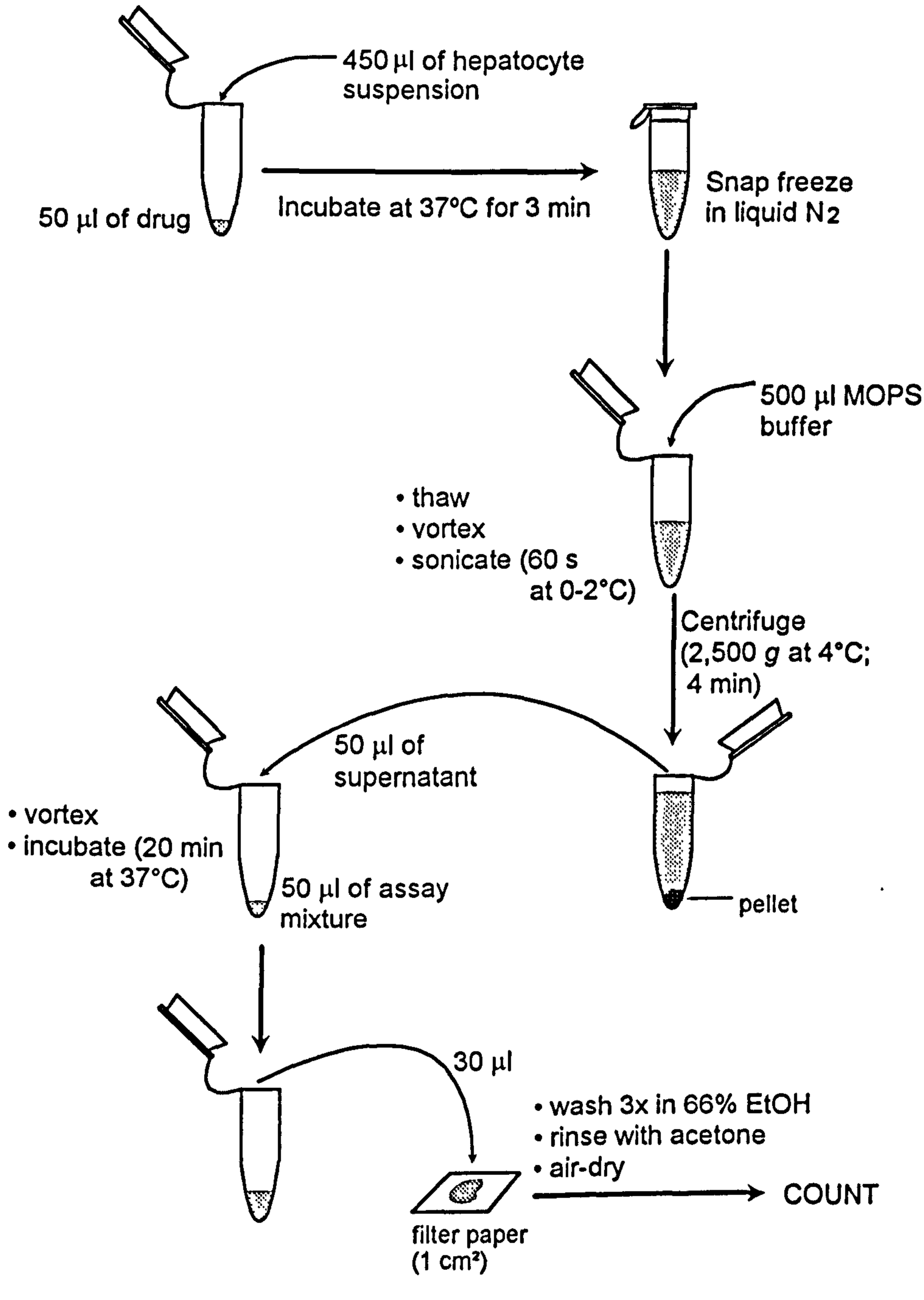


Figure 2.10 Protocol for glycogen phosphorylase a assay.

The total amount of radioactivity present in the assay mixture was measured by spotting 50µl of assay mixture onto a filter paper, allowing to dry and counting as above.

2.9 *Protein assay*

The amount of protein present in the supernatant of freeze thawed cells was measured by the Folin-phenol method (Lowry, 1951) with bovine serum albumin as a standard (as described in section 2.4.2).

2.10 *CALCULATIONS AND STATISTICS:*

An outline of each calculation used in the Results is given below. Some are taken from Gibson and Skett, (1994).

2.11 *Cytochrome P450-Dependent Enzyme Activities*

The amount of metabolite formed can be calculated by direct reference to the standard curve.

Amount of product in incubation = X nmoles

incubation time = 30 minutes

Protein content = Y mg protein

Enzyme activity = X /30Y nmoles/min./mg protein

2.12 How to calculate the volume of the buffer for microsome preparation

liver weight (g) x 5 ml of buffer

2.13 Total Protein (mg)

= volume of buffer x Protein concentration

2.14 Protein (mg/g)

=Total Protein / Liver weight

2.15 Liver weight as % of Body weight

= Liver weight x 100 / Body weight

2.16 Steroid metabolising enzyme activities

The amount of each metabolite of androst-4-dione-3,17-dione expressed as pmole metabolite formed per minute per 10^6 cell or mg protein was determined using the formula shown below:

pmol metabolite / min. /10^6 cell or mg of protein= S × (Cm/Ct) × (1/t) × (1/n) × (1/MW)

Where

S = amount of substrate added (μg)

Cm = d.p.m. metabolite

Ct = d.p.m. total

MW = molecular weight of substrate (286)

t = incubation time (minutes)

n = number of cells (divided by 10^6)

this gives activity in nmoles product / min. / million cells

2.17 Counting number of cells / 1ml (in steroid metabolism)

Using the haemocytometer:

if there are 20 cells in one large square and the viability is 80% then viable cells =

20 x 0.8 = 16 million cells/ml

If the total volume of cell suspension is 30ml, then

∴ Total number of cells = 30×16 = 480 million viable cells

∴ make up to 48ml, to give

The final concentration = 480÷48 = 10 million cells/1ml

2.18 Calculations of glycogen phosphorylase a activity

The activity of glycogen phosphorylase a in a given sample was measured as the amount of ^{14}C-glucose incorporated into glycogen after subtraction of the blank values.

Enzyme activity is expressed as nmol of ^{14}C-glucose 1-phosphate into glycogen/min./mg of protein.

ENZYME ACTIVTY=$(DPM_S-DPM_B)/DPM_T$X [S] X (1/P) X (1/t) X (10,000/3)

where,

DPM_S, DPM_B, DPM_T are the disintegrations/min. for the sample, blank and total amount of radioactivity in the assay respectively.

[S] = substrate (Glc1*P*) concentration (50mM in all the assays)

P = protein conc. of the supernatant

t = incubation time (min)

Results were expressed as percentage increase in phosphorylase *a* activity of appropriate control values.

2.19 STATISTICS

The means and standard deviation were calculated where appropriate. Statistical differences were determined by the ANOVA followed by Dunnet's test and the level of significance set at $P<0.05$. In many cases results were calculated as percentage of relevant control values (as the control values could vary between cell preparations and between experiments) to make understanding of the results easier. In these cases statistics were performed on the untransformed data and these statistics given.

2.20 REAGENTS USED

2.20.1 BUFFERS

To make 1 Molar phosphate Buffer pH 7.4

Make the following solutions:

Solution A: 9.08g of KH_2PO_4 (BDH Chemicals) dissolved in 1 liter of distilled water

Solution B: 11.88g of $Na_2HPO_4.2H_2O$ (Hopkin and Williams) dissolved in 1 liter of distilled water

0.1M solution was prepared by adding 19.6ml of solution A to 80.4ml of solution B and diluting to 1 litre. pH = 7.4

100ml of 0.1M buffer was diluted to 1 litre and 11.5g of potassium chloride was added to give 0.01M buffer (pH=7.4) for use in microsome preparation.

2.20.2 NASH reagent:

75g of ammonium acetate (BDH Chemicals) was dissolved in 500ml distilled water. 1.5ml glacial. acetic acid (M&B) and 1ml acetylacetone (Sigma Chemical Co.Ltd.) was added. This reagent is made just before it is used as it is unstable.

2.20.3 CO-FACTOR MIXTURE FOR ENZYME ASSAYS

For each incubation

3mg $NADP^+$ (Sigma Chemical Co.Ltd.)

3.9 mg Trisodium Isocitrate (Sigma Chemical Co.Ltd.)

dissolved in 100µl $MnCl_2$ solution (6mg/100ml) (BDH Chemicals)

Add 10µl Isocitrate Dehydrogenase (Boehringer Mannheim).

$$\text{Isocitrate} + NADP^+ \rightarrow \alpha\text{-ketoglutarate} + NADPH + H^+ + CO_2$$

2.21 Buffers and physiological solutions:

Perfusion buffers were prepared from a 5×stock solution containing the ingredients listed in 500ml of distilled water.

NaCl	17.1g
$NaHCO_3$	5.25g
HEPES	6.50g
Glucose	5.00g
KCl(10% w / v soln)	8.75ml
$MgSO_{4.7H2o}$	7.25ml
KH_2PO_4	4.00ml

The stock solution was stored at 2 to 4°C and was not used if more than a week old.

2.21.1 Ca^{2+}-Free Krebs-Henseleit solution (with EGTA) -[500ml]

Ca^{2+}-Free buffer was prepared by dissolving 1.9g EGTA in 100ml of the stock solution that has been diluted to 450ml with distilled water. The pH was adjusted to 7.4 with 0.1 M NaOH and the volume made up to 500ml with distilled water.

2.21.2 Ca^{2+} free Buffer (with out EGTA) [100ml]:

Dilute 20ml of stock solution to 100ml with distilled water. Adjust pH to 7.4 with 0.1M NaOH.

2.21.3 Collagenase buffer [100ml]:

As above (section 2.21.2) but containing 4.2 mM $CaCl_2$ (0.42ml of a 1M $CaCl_2$ stock solution / 100 ml of buffer) and the pH adjusted to 7.6. Due to the high concentration of Ca^{2+} precipitation of calcium salts may occur in the collagenase buffer. This is undesirable and to avoid precipitation, the $CaCl_2$ solution was added gradually to the solution while stirring.

2.21.4 Washing buffer

As in (section 2.21.2) without EGTA and containing 1.5 mM $CaCl_2$ (0.75 ml of 1M $CaCl_2$ in 500ml), adjusted at pH 7.4.

2.21.5 Buffer and radioactive mixture for glycogen phosphorylase *a* assay

2.21.5.1 MOPS Buffer

	(g /100ml)
MOPS	2.09
NaF	0.84
NaEDTA.....	1.12
Dithiothreitol (DTT)	0.154
Dist. water ..... to	100ml

Adjust pH to 6.5 with 0.1M KOH. Store at 2 to 4 °C. Discard if more than a week old.

2.21.5.2 Radioactive assay mixture

	(mg/10ml)
Caffeine	1.00mM
Glycogen	2.00%
α-D-Glucose-1 phosphate	336.3mg
α-D-[U-^{14}Glucose-1 phosphate	25.0µl
Distilled water	10.0ml

- Dissolve the glycogen, caffeine, and unlabelled Glc1P in diluted MOPS buffer (1:1) with GENTLE shaking.

- Adjust pH to 6.5 with 0.1 N HCL, the labeled Glc1P added and mixed thoroughly.

Transfer 1-ml aliquots to microcentrifuge tubes and store at -20 °C. (will keep for 6 months).

2.22 - SOURCES OF HORMONES,DRUGS AND REAGENTS

Hormones, chemical and reagents are listed according to the manufacturer.

Sigma Chemical Co.Ltd., St.Louis, MO, USA:

MOPS

DL-dithiothreitol

Caffeine

Rabbit liver glycogen type111

HEPES

Insulin (porcine)

EGTA

Streptozotocin

Bovine serum albumin (Fraction V)

4-Androstene-3,17-dione

α-D-Glucose-1-phosphate

Glucose Assay Kit

Glucose / Urea Nitrogen

combined standards set

Glucagon

Insulin Bovine

$CuSO_4.H_2O$

Nicotinamide

Semicarbazide

Aminopyrine

Hopkins & Williams, Essex, England:

Sodium fluoride

Disodium EDTA

British Drug Houses, Poole, England

Folin-Ciocalteu' phenol reagent

glycine

Potassium Tartrate

Sodium Hydrosulphite

Magnesium Chloride

Aniline

Trichloroacetic Acid

4-Aminophenol

Zinc Sulphate

Calcium Chloride

Amersham International p.l.c., Bucks, England:

[U-^{14}C]-D-Glucose-1 phosphate

Potassium salt

[4-^{14}C] androst-4-ene-3,17-dione

Boehringer, Mannheim, Germany:

Collagenase A from Clostridium histolyticum

Keto-Diabur Test 5000 strips

GIBCO BRL Limited

Ham's F-10 Culture medium ,

Williams' E medium

Penicillin / Streptomycin

. Fisons, Loughborough, England

NaCl, NaOH, KCl, $MgSO_4$, $NaHCO_3$, KH_2PO_4,Glucose

Kodak, France

X Ray film (X-Omat)

Devleloper (DX 24)

Fixer (FX-40)

RESULTS AND DISCUSSIONS

3. *IN-VIVO* EXPERIMENTS

The tables and figures represent the results which were collected from each experimental run and the statistical analysis of the results were from 9 normal, healthy rats treated with *Artemisia* extract, 6 diabetic rats and 6 diabetic rats treated with *Artemisia* extract. The results, in general, are presented for individual rats as each animal acted as its own control during the experiments.

3.1 Normal Rats treated with Artemisia *(17 days):*

This experiment was to see if *Artemisia* extract had any observable effects on healthy rats such as changes in body weight, food and fluid intake, urine volume, urine glucose concentrations and faeces output. The results of this experiment are shown for normal rats treated with *Artemisia* extract as follows:

All 9 rats showed normal growth rate. In summary, body weight increased over a 7-days period at a fairly constant rate of 27.2 ± 7.1g before treatment and 28.2 ± 6.9g after treatment (Figure 3.1). Faeces output was not significantly different ($p>0.05$) - 9.69 ± 4.74g before treatment and 6.99 ± 1.96g after treatment (Table 3.1). Food intake did not alter significantly after introduction of the *Artemisia* extract except for rats 3 and 9, which appeared to have a low food intake temporarily upon being in giving the *Artemisia* extract but the feeding habit became normal after 1-2 days. Overall, *Artemisia* had no measurable effect on food intake - 19.5 ± 6.75g before treatment and 22.3 ± 5.86g after treatment (Figure 3.2). Fluid intake in all 9 rats was unchanged by treatment except in rats 4, 5 and 6 where it was reduced slightly in the presence of *Artemisia* extract on the first day of giving *Artemisia.* This is probably due to a taste factor - *Artemisia* extract is bitter. Overall, however, *Artemisia* extract had no effect on fluid intake - 22.4 ± 6.2ml before treatment and 24.89 ± 5.77ml after treatment (Figure 3.3).

Urine volume significantly increased from 7.11 ± 3.16 ml to 9.3 ± 3.05ml ($p<0.05$) after treatment with *Artemisia* extract (Figure 3.4). This was seen in all rats but is particularly apparent in rats 1-6 with little change in rats 7 and 8. Despite the unchanged intake of fluid as seen above, it is seen that *Artemisia* extract appeared to act

Figure 3.1 The effect of *Artemisia judaica* extract on body weight of control male rats.

(↑) = Start of *Artemisia* treatment (note : treatment started on day 11 in experiment 1, (a) and day 7 in experiments 2&3, (b,c)).

Results for individual rats are shown.

(o) = rat (1, 4, 7); (•) = rat (2, 5, 8); (∇) = rat (3, 6, 9)

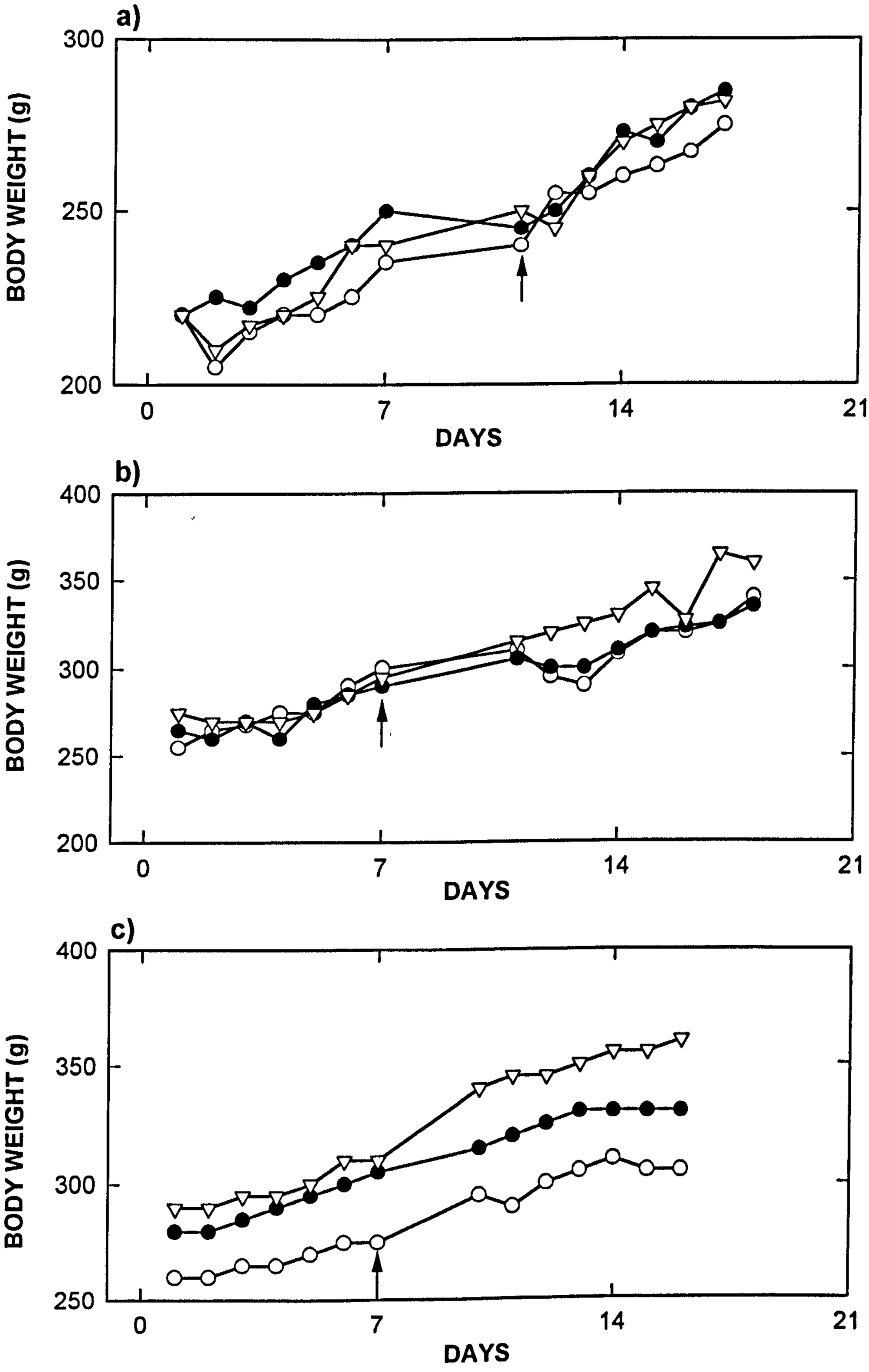

a)
300
250
200
BODY WEIGHT (g)
0
7
14
21
DAYS
b)
400
350
300
250
200
BODY WEIGHT (g)
0
7
14
21
DAYS
c)
400
350
300
250
BODY WEIGHT (g)
0
7
14
21
DAYS

Figure 3.2 The effect of *Artemisia judaica* extract on food intake of control male rats.

(↑) = Start of *Artemisia* treatment (note : treatment started on day 11 in experiment 1, (a) and day 7 in experiments 2&3 (b,c)).

Results for individual rats are shown.

(o) = rat (1, 4, 7); (•) = rat (2, 5, 8); (∇) = rat (3, 6, 9)

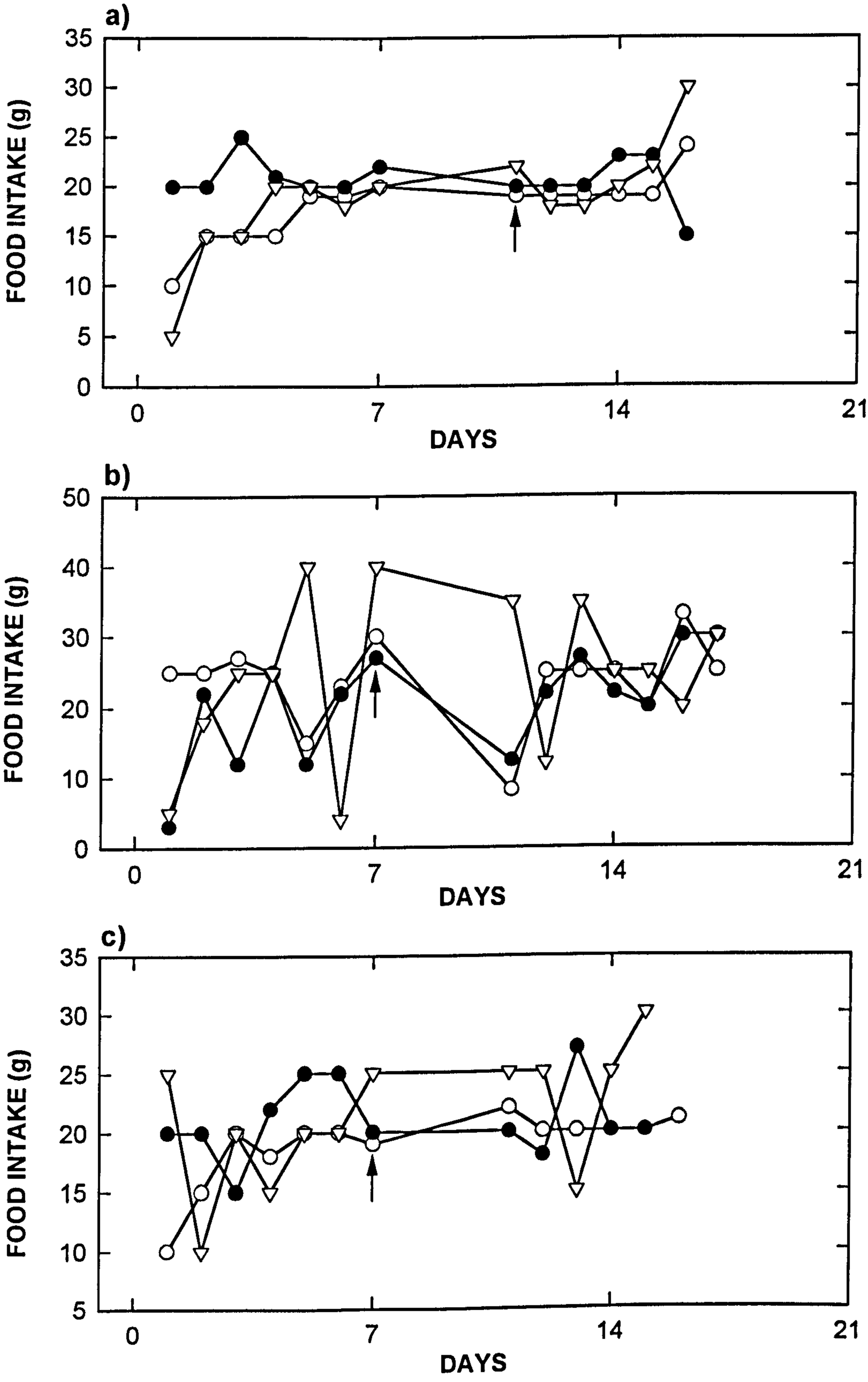

a)
FOOD INTAKE (g)
35
30
25
20
15
10
5
0
0
7
14
21
DAYS
b)
FOOD INTAKE (g)
50
40
30
20
10
0
0
7
14
21
DAYS
c)
FOOD INTAKE (g)
35
30
25
20
15
10
5
0
7
14
21
DAYS

Figure 3.3 The effect of *Artemisia judaica* extract on fluid intake of control male rats.

(↑) = Start of *Artemisia* treatment (note : treatment started on day 11 in experiment 1, (A) and day 7 in experiments 2&3 (c,b)).

Results for individual rats are shown.

(o) = rat (1, 4, 7); (•) = rat (2, 5, 8); (∇) = rat (3, 6, 9)

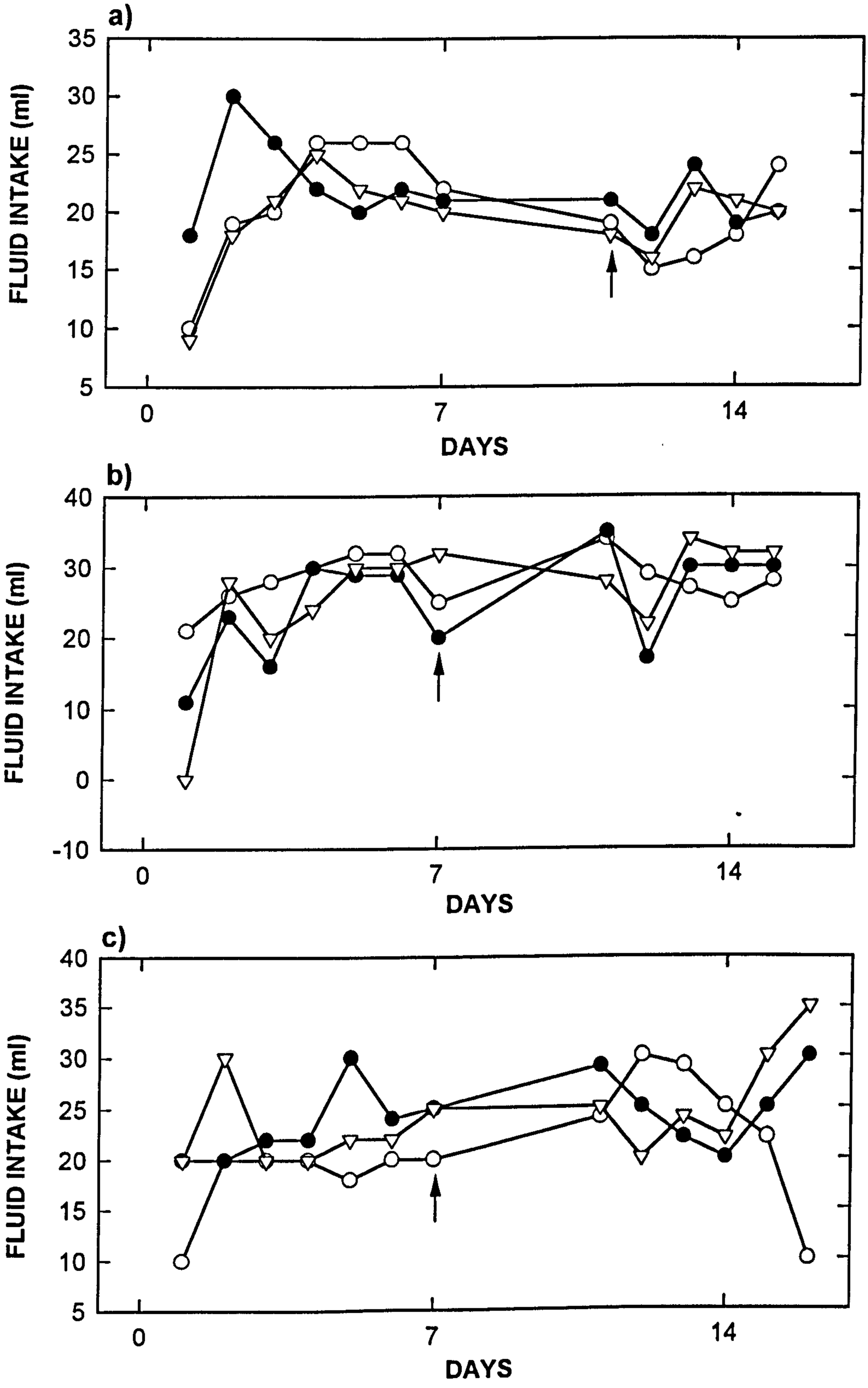
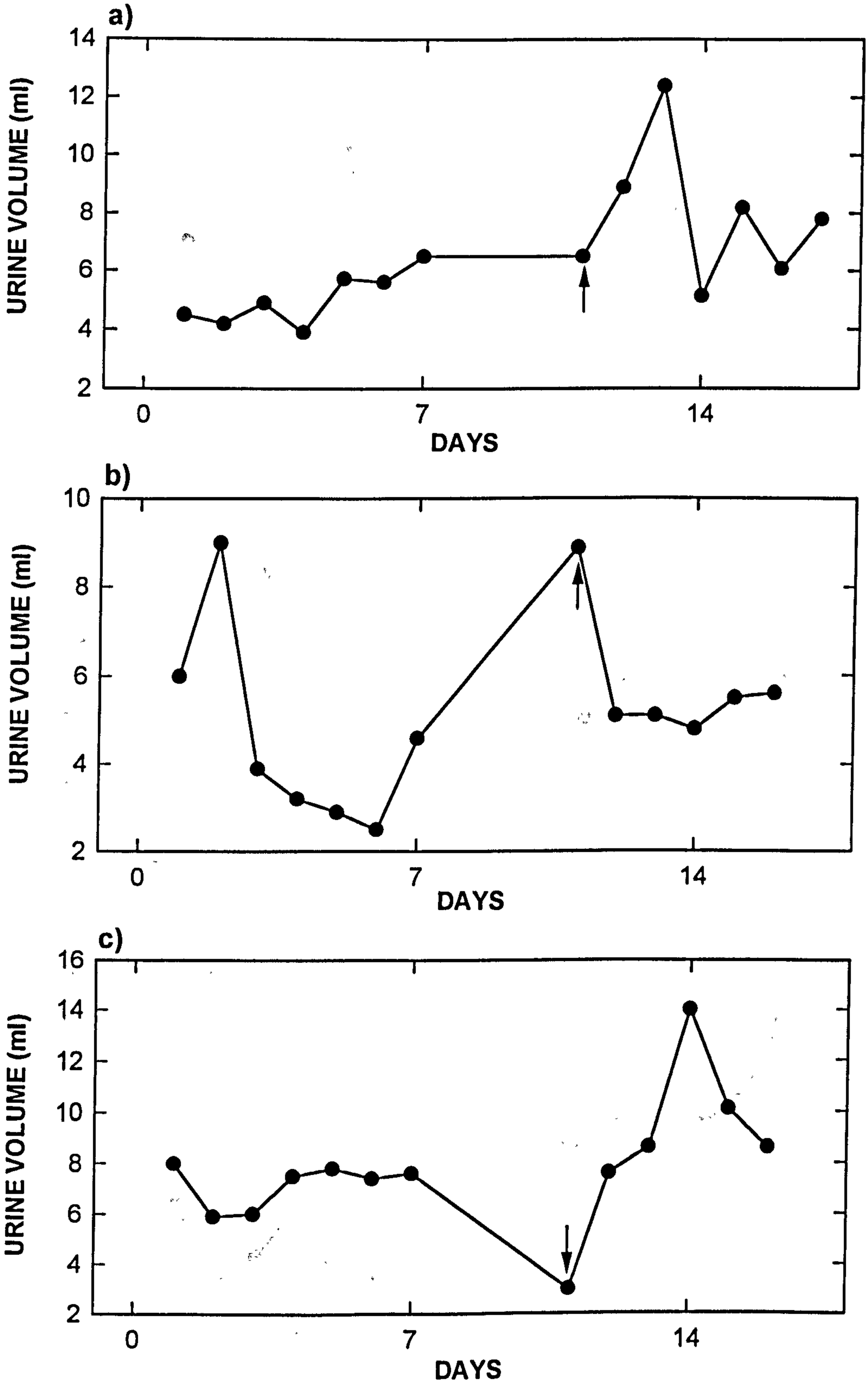
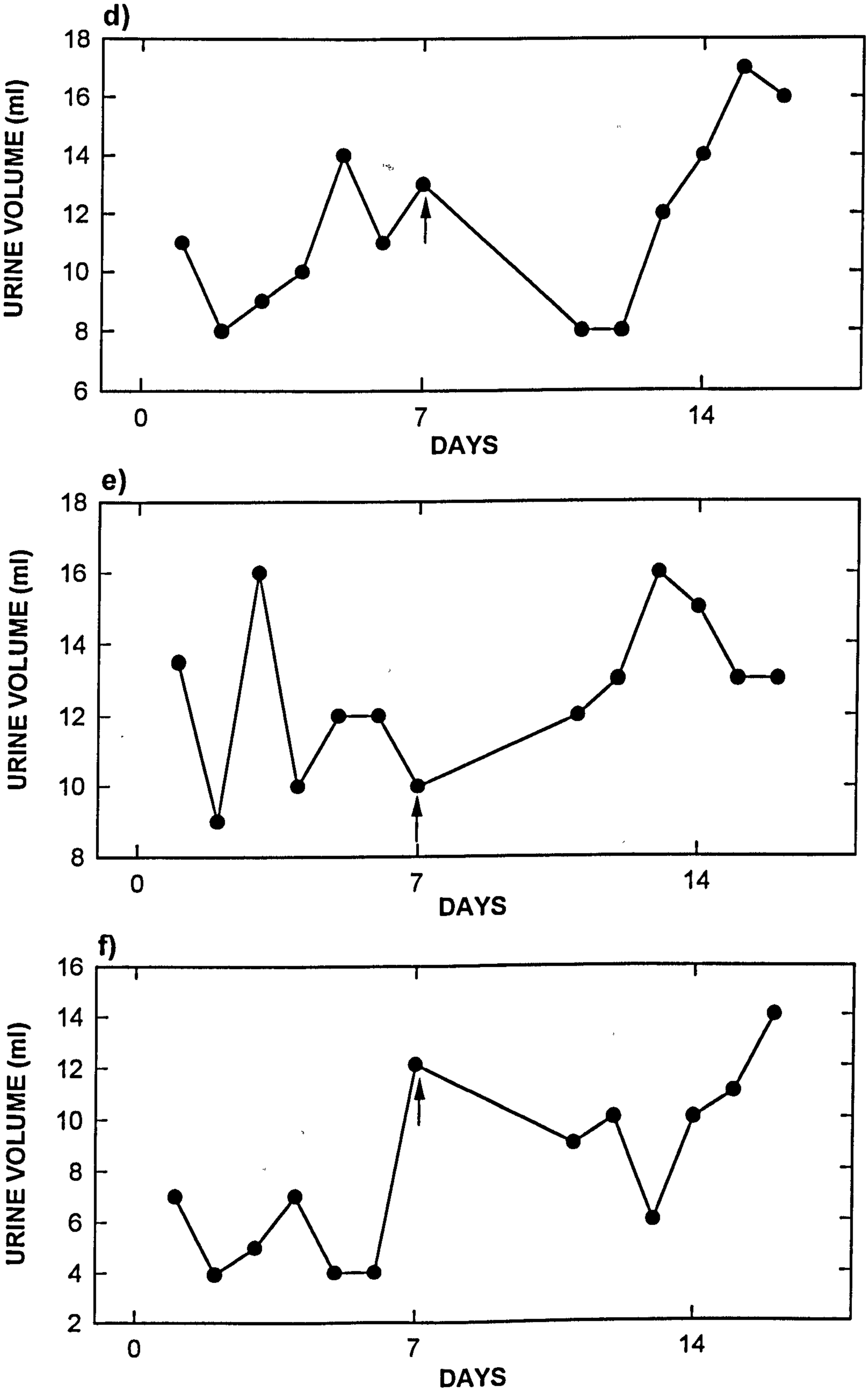

a)
FLUID INTAKE (ml)
35
30
25
20
15
10
5
0
7
14
DAYS
b)
FLUID INTAKE (ml)
40
30
20
10
0
-10
0
7
14
DAYS
c)
FLUID INTAKE (ml)
40
35
30
25
20
15
10
5
0
7
14
DAYS

Figure 3.4 The effect of Artemisia judaica extract on urine volume of control male rats.
(↑) = Start of *Artemisia* treatment (note : treatment started on day 11 in experiment 1, (a) and day 7 in experiments 2&3, (b,c)):
Results for individual rats are shown.

a = rat1, b = rat2, c = rat3, d = rat4, e = rat5, f = rat6, g = rat7, h = rat8 & i = rat9


a)
URINE VOLUME (ml)
14
12
10
8
6
4
2
0
7
14
DAYS
b)
URINE VOLUME (ml)
10
8
6
4
2
0
7
14
DAYS
c)
URINE VOLUME (ml)
16
14
12
10
8
6
4
2
0
7
14
DAYS


d)
18
16
14
12
10
8
6
URINE VOLUME (ml)
0
7
14
DAYS
e)
18
16
14
12
10
8
URINE VOLUME (ml)
0
7
14
DAYS
f)
16
14
12
10
8
6
4
2
URINE VOLUME (ml)
0
7
14
DAYS

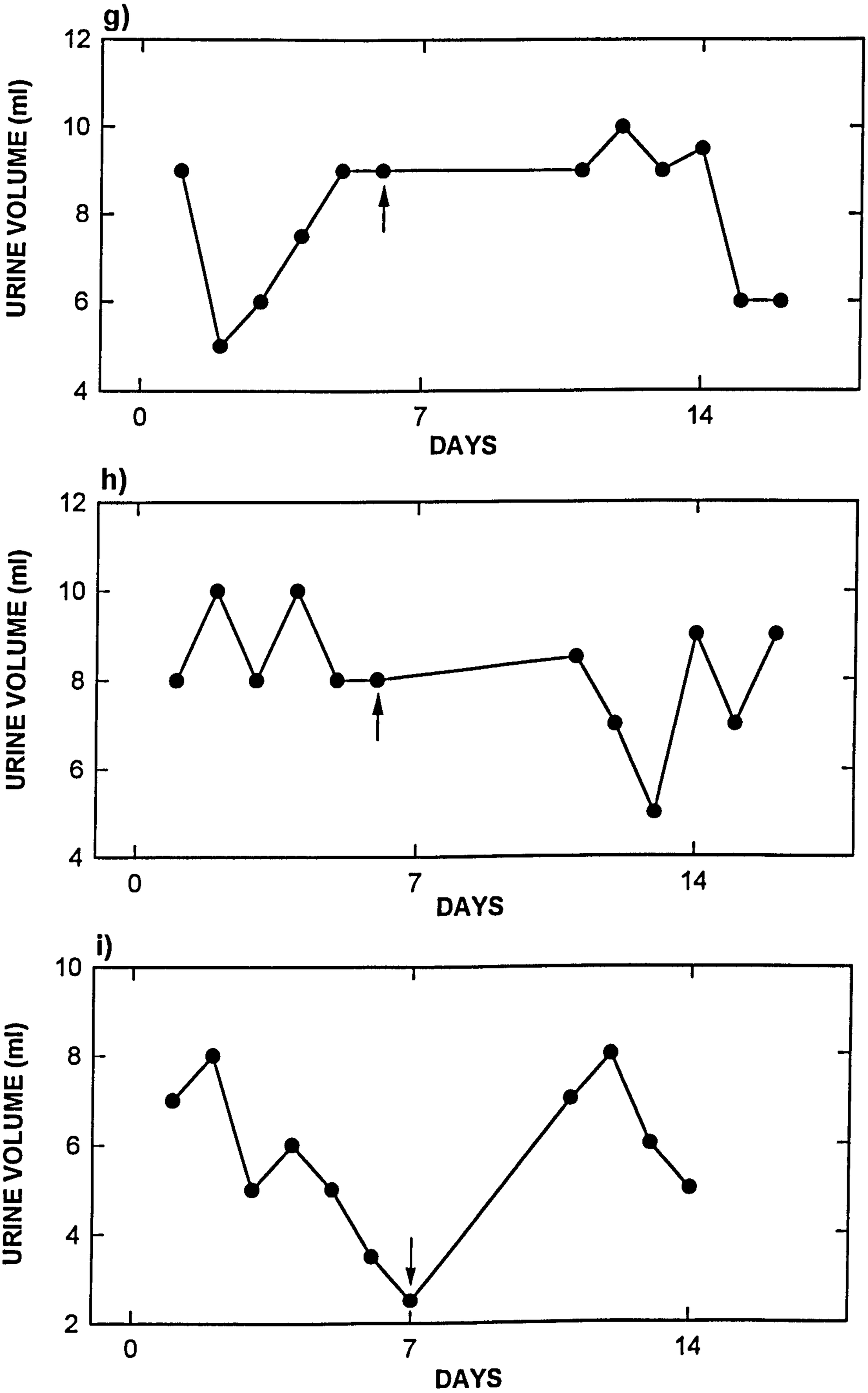
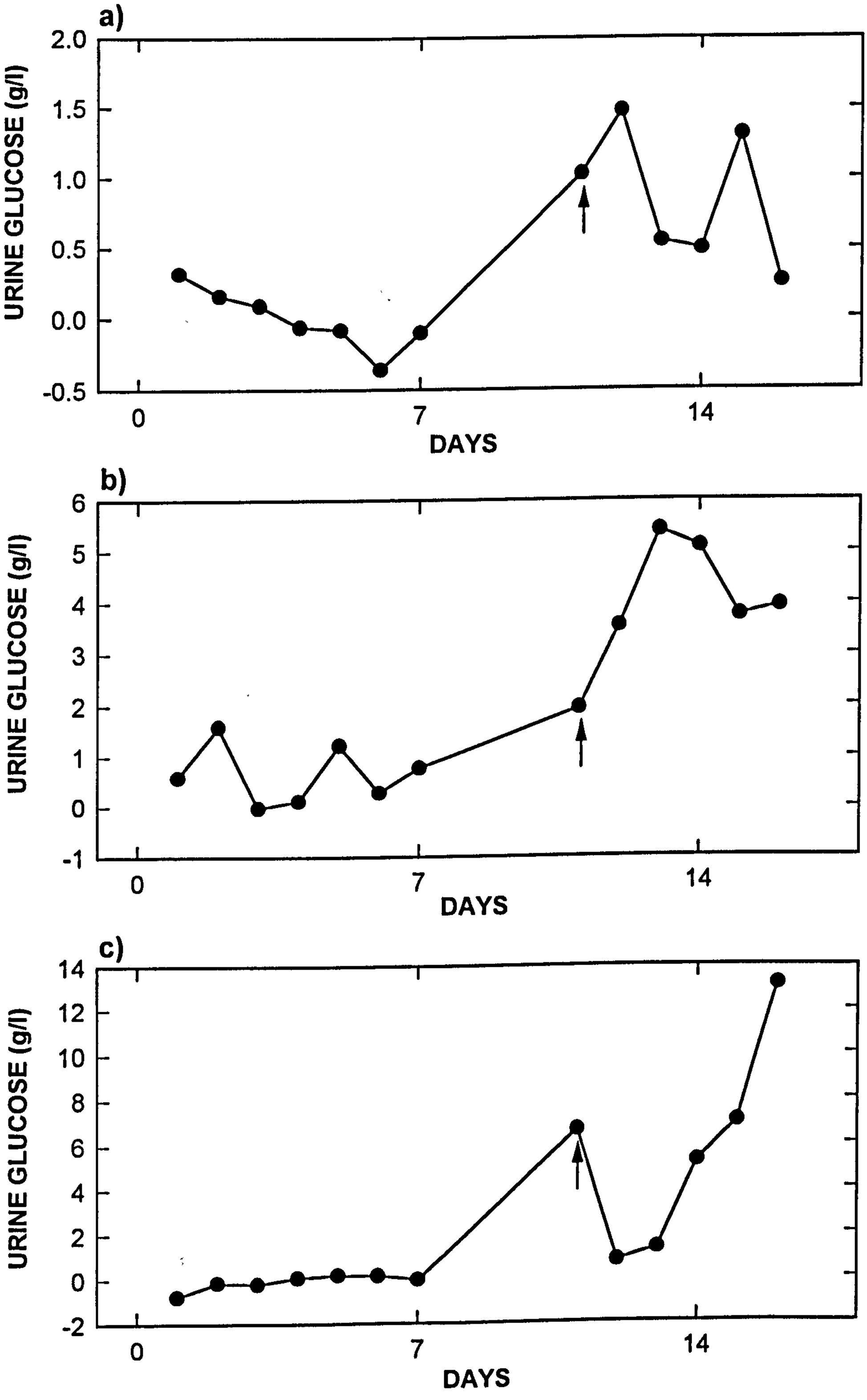

g)
12
10
8
6
4
URINE VOLUME (ml)
0
7
14
DAYS
h)
12
10
8
6
4
URINE VOLUME (ml)
0
7
14
DAYS
i)
10
8
6
4
2
URINE VOLUME (ml)
0
7
14
DAYS

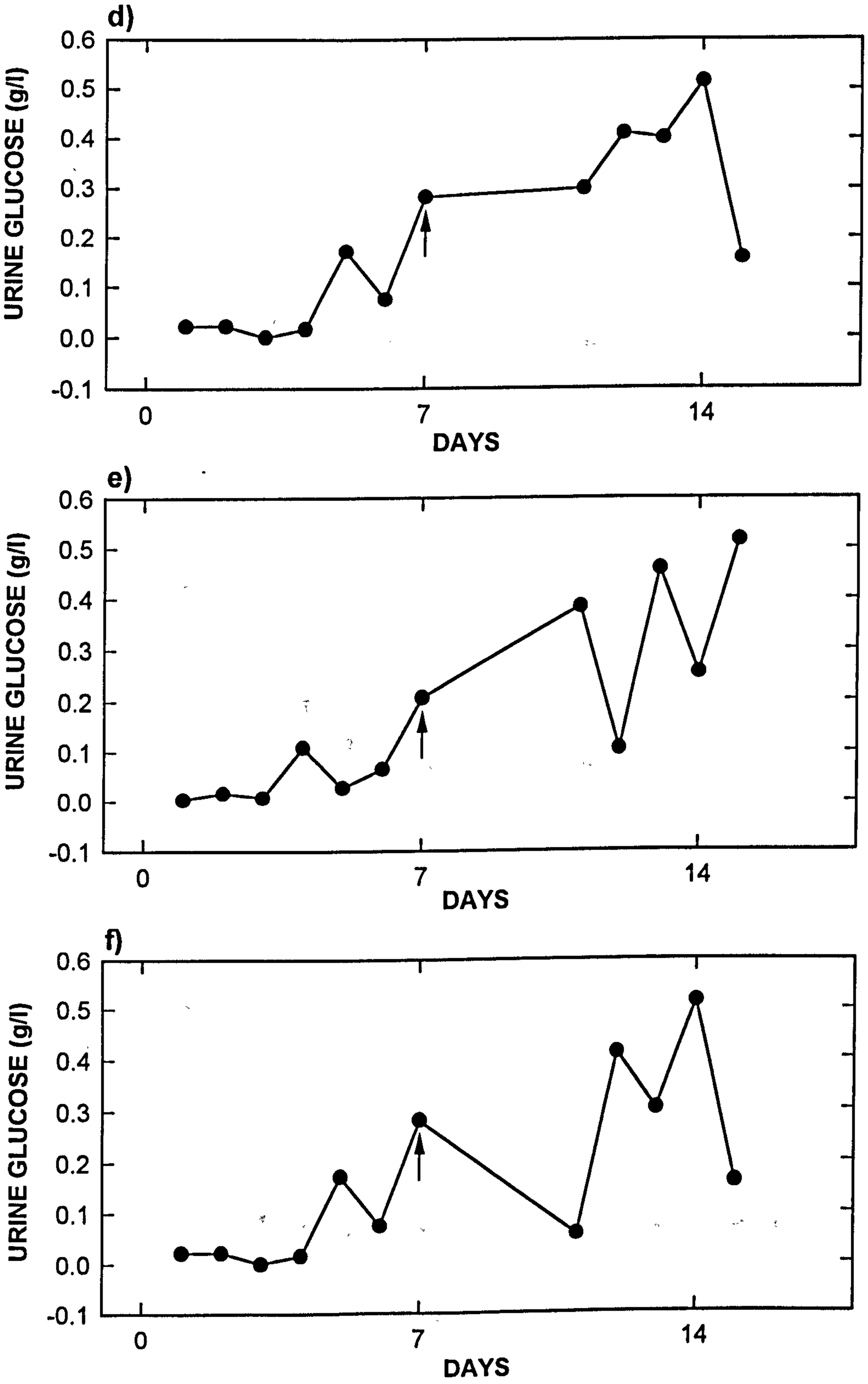
Figure 3.5 The effect of *Artemisia judaica* extract on urine glucose of control male rats.

(↑) = Start of *Artemisia* treatment(note : treatment started on day 11 in experiment 1, (a) and day 7 in experiments 2&3, (b,c)).

Results for individual rats are shown.

a = rat1, b = rat2, c = rat3, d = rat4, e = rat5, f = rat6, g = rat7, h = rat8 & i = rat9


a)
URINE GLUCOSE (g/l)
2.0
1.5
1.0
0.5
0.0
-0.5
0
7
14
DAYS
b)
URINE GLUCOSE (g/l)
6
5
4
3
2
1
0
-1
0
7
14
DAYS
c)
URINE GLUCOSE (g/l)
14
12
10
8
6
4
2
0
-2
0
7
14
DAYS


d)
URINE GLUCOSE (g/l)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
0
7
14
DAYS
e)
URINE GLUCOSE (g/l)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
0
7
14
DAYS
f)
URINE GLUCOSE (g/l)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
0
7
14
DAYS

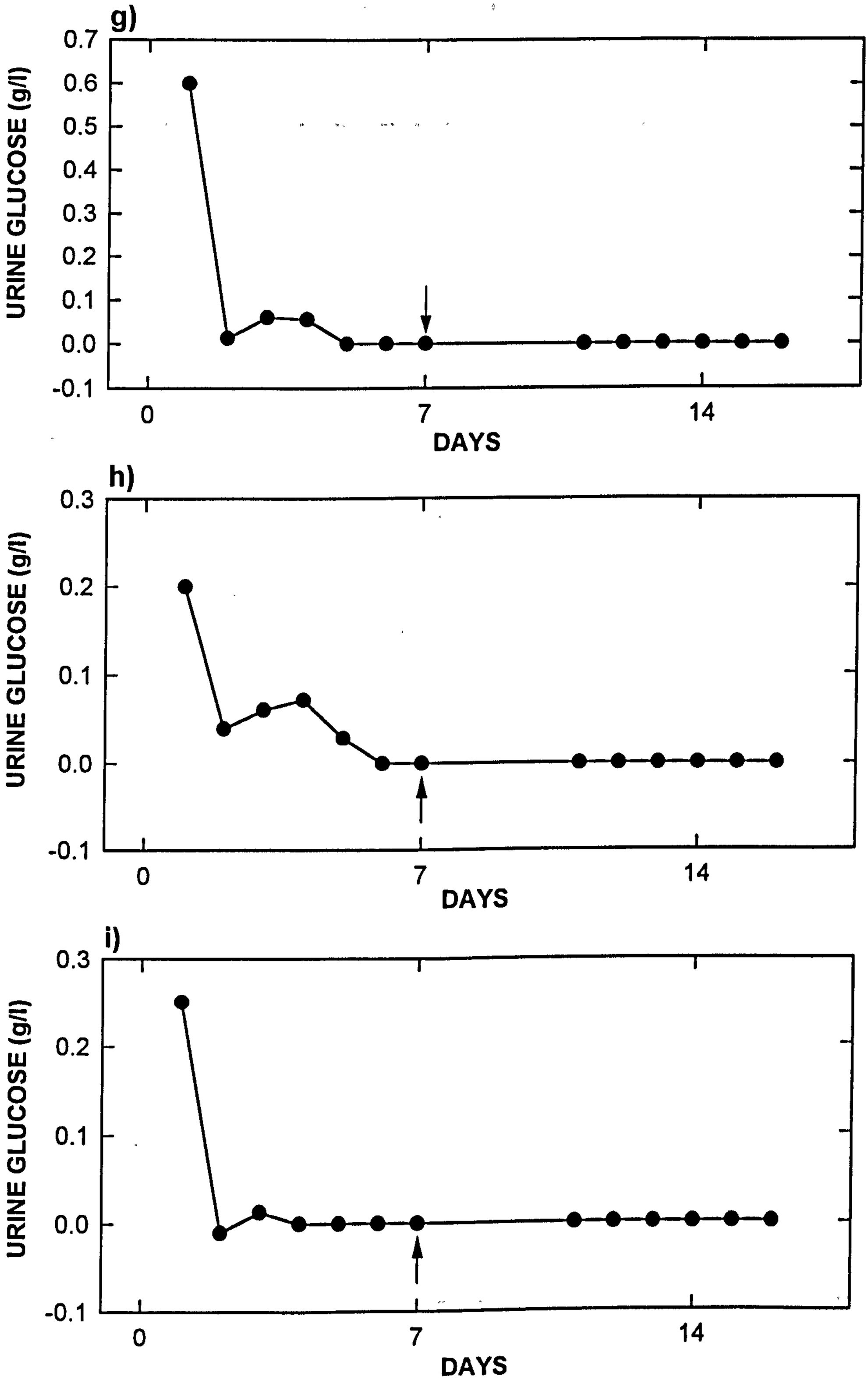

g)
URINE GLUCOSE (g/l)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
0
7
14
DAYS
h)
URINE GLUCOSE (g/l)
0.3
0.2
0.1
0.0
-0.1
0
7
14
DAYS
i)
URINE GLUCOSE (g/l)
0.3
0.2
0.1
0.0
-0.1
0
7
14
DAYS

Table 3.1: Faeces (g) weight of normal rat treated with *Artemisia judaica* from day 11 in experiment 1 and in day 7 in experiment 2 & 3 , (n.d. = not determined)

DAY	RAT1	RAT2	RAT3	RAT4	RAT5	RAT6	RAT7	RAT8	RAT9
1	3.42	4.98	2.87	4.10	7.3	8.00	9.30	2.88	3.48
2	5.40	4.30	2.50	5.80	7.60	6.40	12.25	8.30	3.92
3	4.48	5.42	5.59	5.90	9.50	6.50	12.0	8.61	10.0
4	3.46	4.22	4.13	7.80	9.20	6.20	8.40	10.3	10.01
5	5.73	3.66	7.32	6.50	6.60	6.70	11.33	9.60	9.90
6	5.43	4.72	5.68	8.90	9.40	9.60	10.43	8.96	9.34
7	4.01	4.02	4.29	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
11	5.62	6.84	6.62	4.00	5.50	5.00	8.22	12.5	12.1
12	5.13	7.86	5.30	5.50	6.50	7.00	12.7	8.61	10.0
13	5.04	7.05	5.17	4.50	5.00	7.10	5.92	6.57	8.57
14	5.14	6.8	5.22	8.4	7.4	8.5	9.95	9.03	9.73
15	5.14	6.80	5.22	5.90	5.40	8.80	6.89	7.72	6.24
16	6.08	6.29	4.87	5.50	7.30	7.50	8.65	6.87	7.79
17	4.20	6.80	4.83	n.d.	n.d.	n.d.	10.0	8.65	6.89

as a mild diuretic There were no detectable ketones in the urine of any of the animals whereas urine glucose concentrations were significantly increased in rats 1-6 ($p<0.05$) but not in rats 7-9 (Figure 3.5).

Artemisia extract, therefore, has little effect on normal animals except for a slight diuresis. This is probably related to the apparent appearance of glucose in the urine of rats treated with the extract. Analysis of a sample of *Artemisia* extract showed that it did indeed contain substances giving a positive reaction in the glucose assay (results not shown). This is unlikely to be glucose itself as this would be retained in the body and used as an energy source but, perhaps, a non-metabolisable sugar derivative which is directly excreted in the urine and, thus, acts as an osmotic diuretic (e.g. mannitol).

3.2 Diabetic Rats (17 days):

Before testing the effect of the *Artemisia* extract on diabetic rats an experiment was carried out to determine any changes in the measured parameters in rats suffering from diabetes mellitus. Animals were monitored for a 7 day period before being made diabetic so that each animal acted as its own control.

On administration of STZ a pronounced decrease in body weight of all rats was seen especially in rat 2 (around 20g weight loss) and rat 6 (30g weight loss) (Figure 3.6). Despite this weight loss all rats had an increased food intake (hyperphagia) (18.93±7.92g) in control animals and 37.20±8.47g in treated animals ($p<0.05$)) (Figure 3.7). Fluid intake significantly increased in all rats after induction of diabetes from 21.05±9.10ml to 244.40±50.84ml ($p<0.05$) after treatment with STZ (Figure 3.8) and urine volume also showed a large increase in all rats from 6.21±3.18ml to 200.0±39.0ml ($p<0.05$) after treatment with STZ (Figure 3.9). Faeces weight only showed an increase from 6.48±2.30g to 14.65±3.19g ($p<0.05$) after induction of diabetes (Table 3.2). All urine samples showed glycosuria (Figure 3.10) and all tested positive for ketones after induction of diabetes.

Figure 3.6 The effect of STZ-induced diabetes on body weight.

(↑) = Treatment with STZ (65mg / kg).

Results for individual rats are shown in graphs a and b as.

o = rat (1, 4); • = rat (2, 5); ∇ = rat (3, 6) from two separate experiments (a,b)

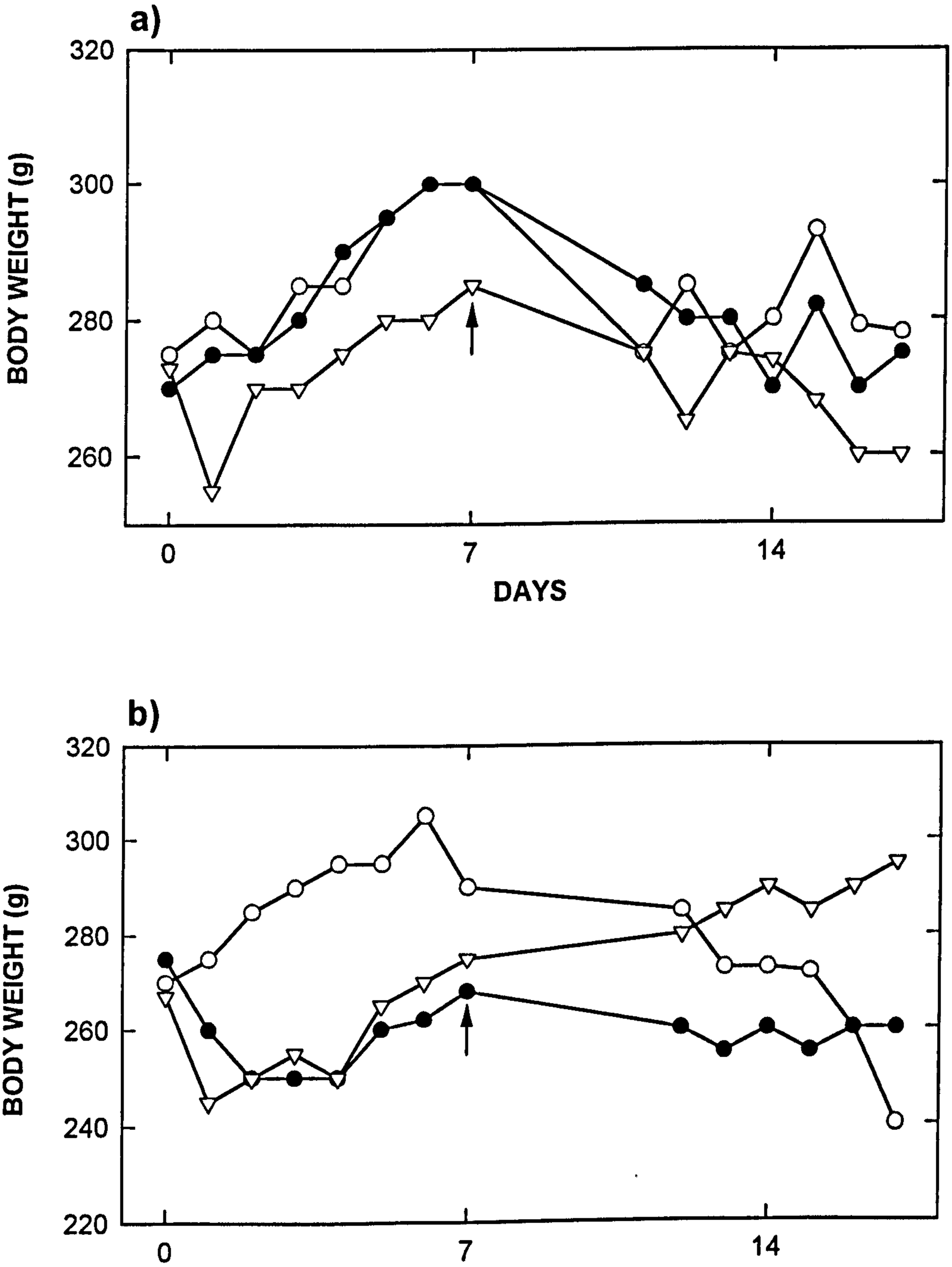
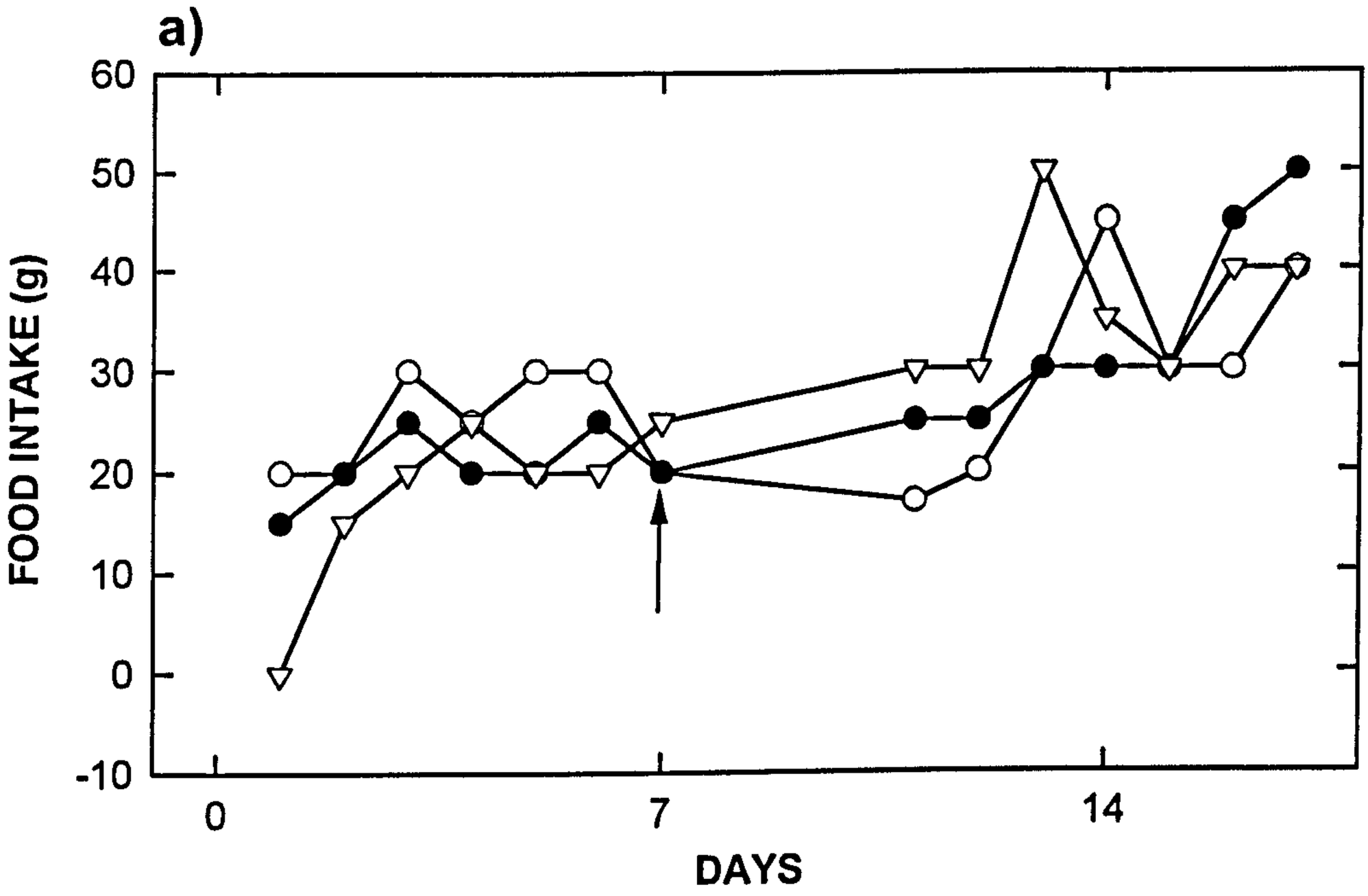

a)
320
300
280
260
BODY WEIGHT (g)
0
7
14
DAYS
b)
320
300
280
260
240
220
BODY WEIGHT (g)
0
7
14
DAYS

Figure 3.7 The effect of STZ-induced diabetes on food intake.

(↑) = Treatment with STZ (65mg / kg).

Results for individual rats are shown in graphs a and b as.

o= rat (1, 4); • = rat (2, 5); ∇ = rat (3, 6)


a)
60
50
40
30
20
10
0
-10
FOOD INTAKE (g)
0
7
14
DAYS

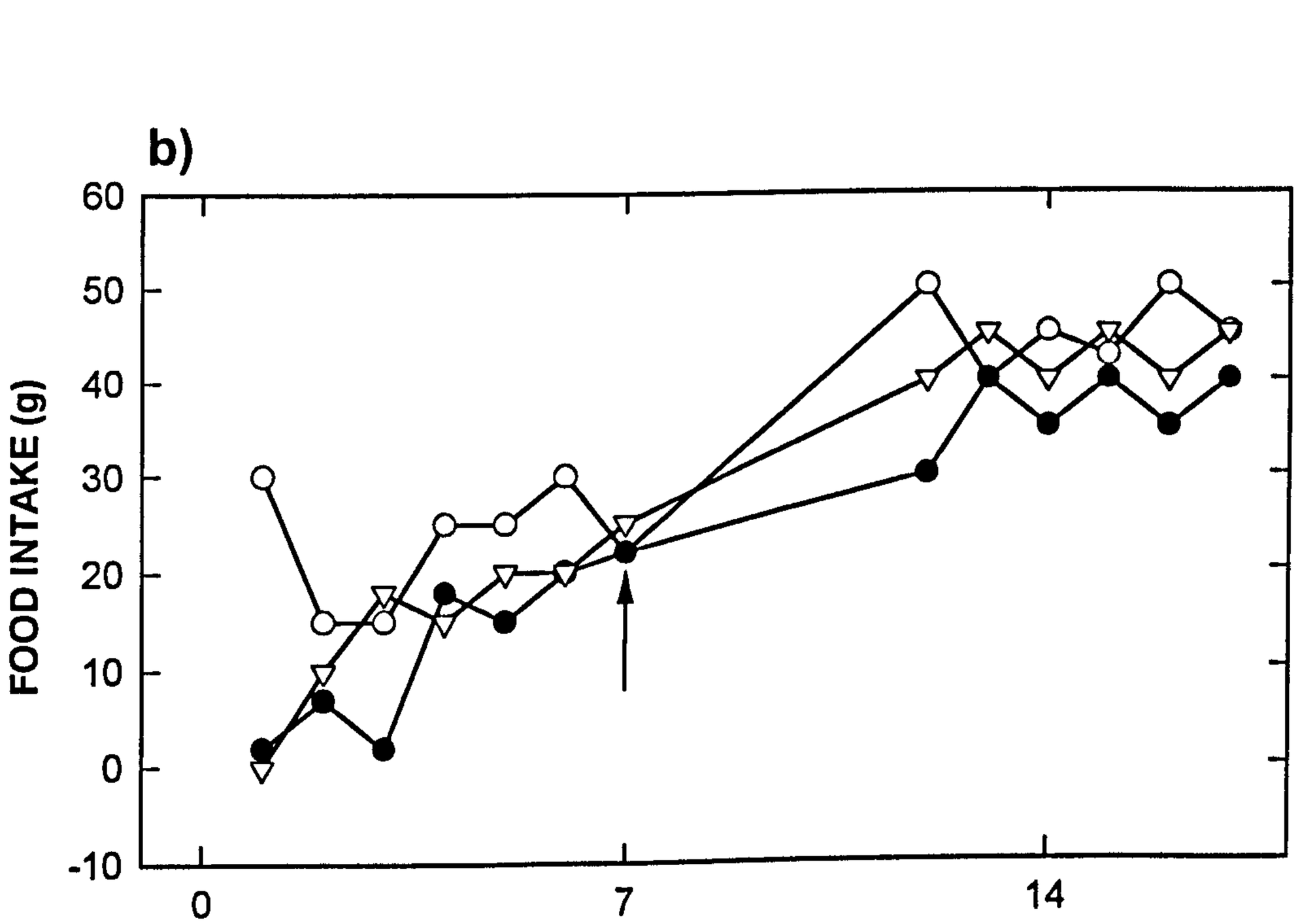
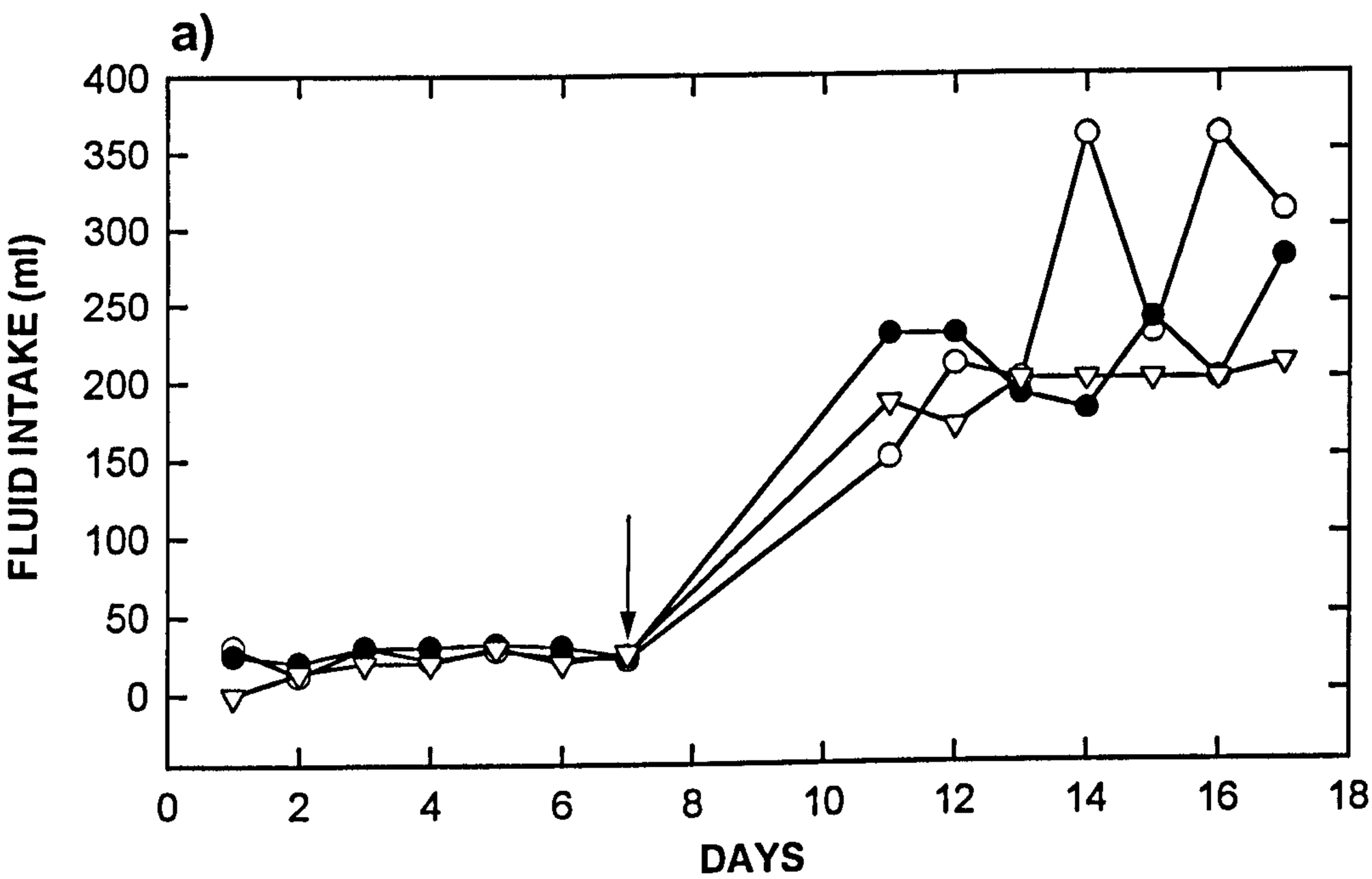

b)
60
50
40
30
20
10
0
-10
FOOD INTAKE (g)
0
7
14
DAYS

Figure 3.8 The effect of STZ-induced diabetes on fluid intake.

. (↑) = Treatment with STZ (65mg / kg).

Results for individual rats are shown in graphs a and b as.

o= rat (1, 4); • = rat (2, 5); ∇ = rat (3, 6)


a)
400
350
300
250
200
150
100
50
0
FLUID INTAKE (ml)
0
2
4
6
8
10
12
14
16
18
DAYS

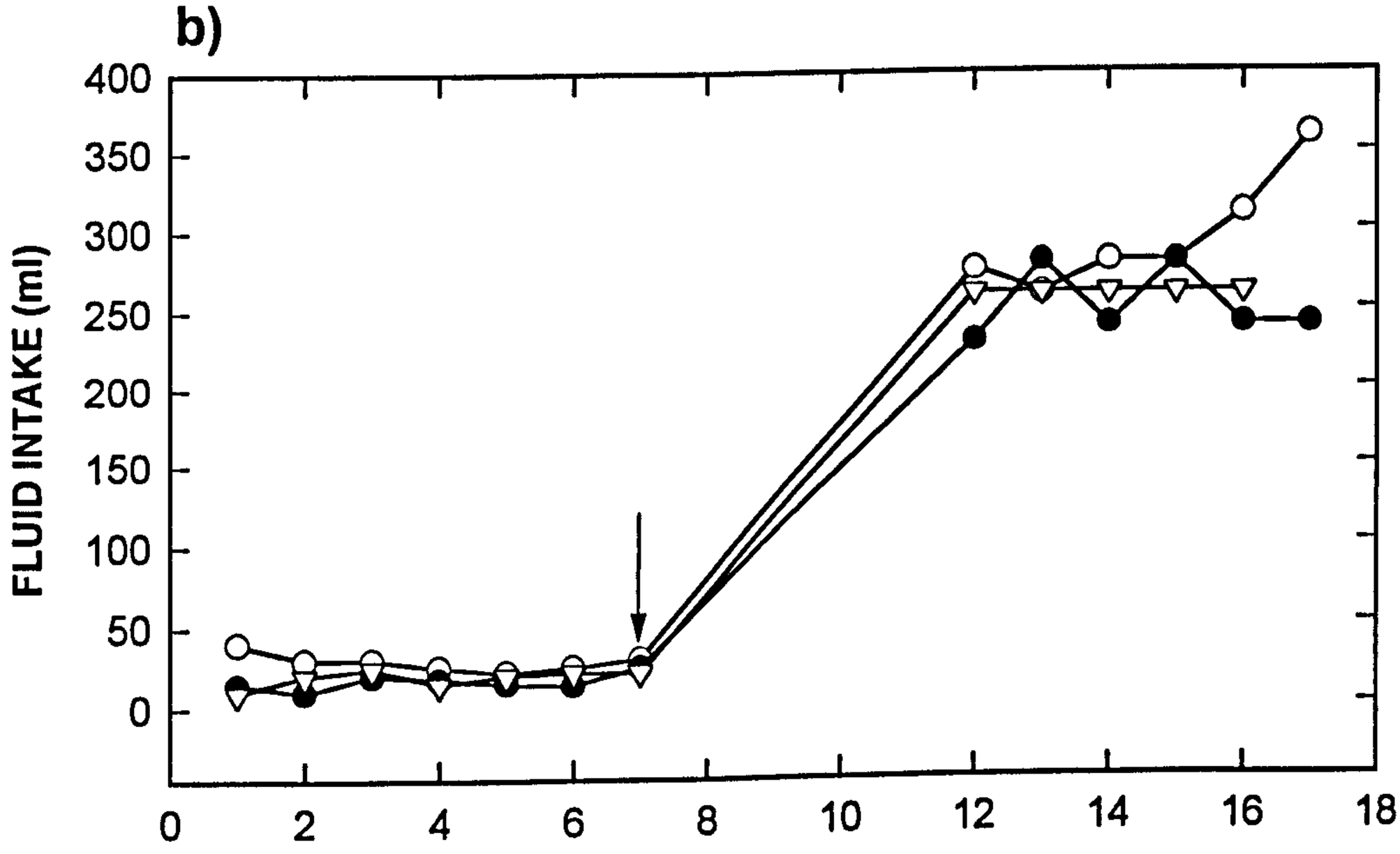

b)
400
350
300
250
200
150
100
50
0
FLUID INTAKE (ml)
0
2
4
6
8
10
12
14
16
18
DAYS

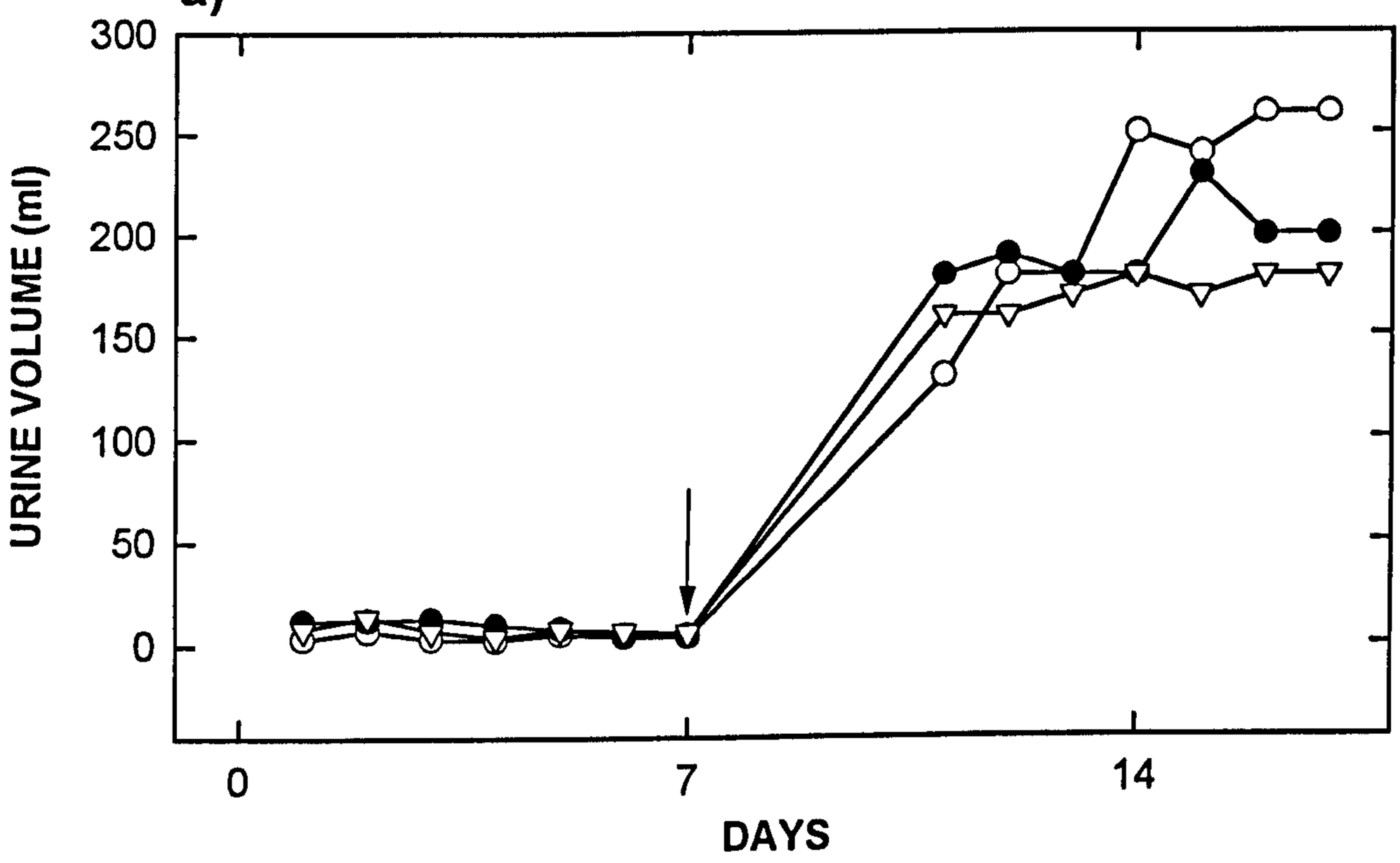
Figure 3.9 The effect of STZ-induced diabetes on urine volume.

(↑) = Treatment with STZ (65mg / kg).

Results for individual rats are shown in graphs a and b as.

o= rat (1, 4); • = rat (2, 5); ∇ = rat (3, 6)

a)


300
250
200
150
100
50
0
URINE VOLUME (ml)
0
7
14
DAYS

b)

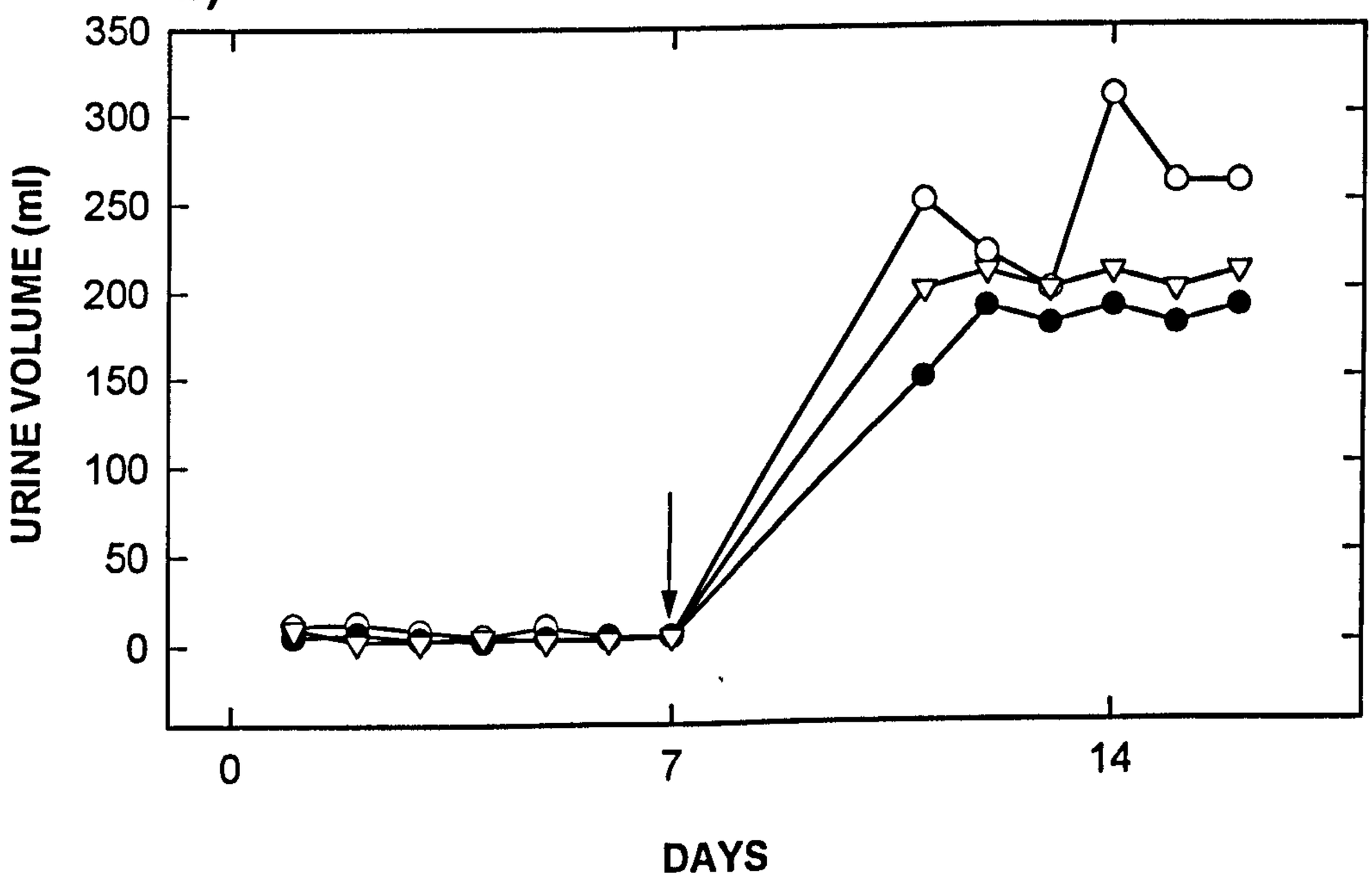

350
300
250
200
150
100
50
0
URINE VOLUME (ml)
0
7
14
DAYS

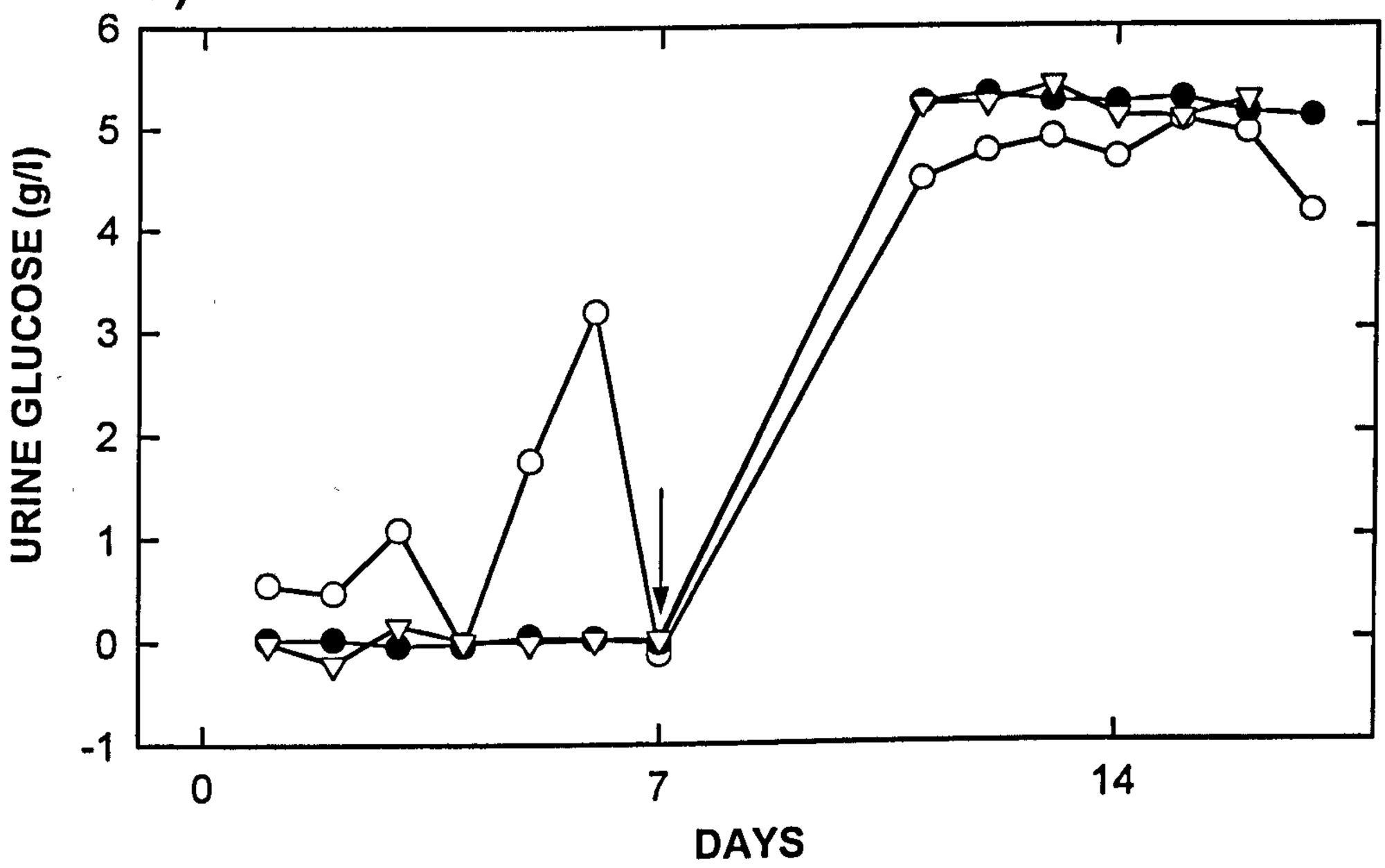
Figure 3.10 The effect of STZ-induced diabetes on urine glucose.

(↑) = Treatment with STZ (65mg / kg).

Results for individual rats are shown in graphs a-f as.

o= rat (1, 4); • = rat (2, 5); ∇ = rat (3, 6)

a)


URINE GLUCOSE (g/l)
6
5
4
3
2
1
0
-1
0
7
14
DAYS

b)

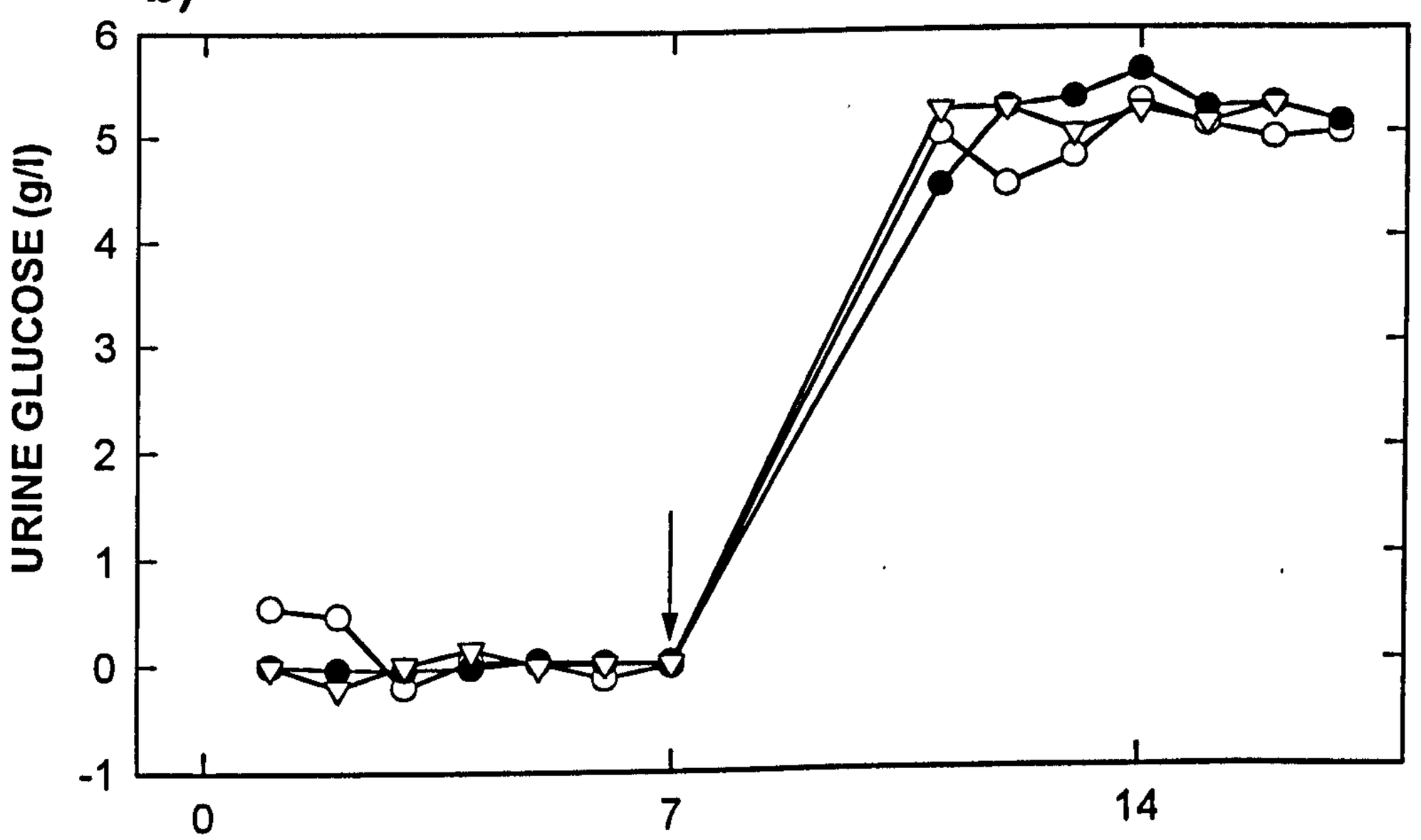

URINE GLUCOSE (g/l)
6
5
4
3
2
1
0
-1
0
7
14
DAYS

Table 3.2 Faeces (g) of diabetic rat on day 7 STZ was given (n.d. = not determined).

DAYS	RAT1	RAT2	RAT3	RAT4	RAT5	RAT6
1	11.2	3.40	1.50	5.00	2.20	2.40
2	7.40	7.00	4.20	7.10	9.50	13.5
3	10.5	7.50	7.00	7.50	4.00	6.00
4	5.50	6.50	4.00	8.50	4.00	2.50
5	7.50	7.50	8.10	10.0	4.00	2.50
6	11.5	5.50	9.20	8.80	4.90	7.30
7	5.50	6.90	8.00	n.d	5.00	5.00
11	11.9	11.0	14.6	n.d.	n.d.	n.d.
12	10.3	15.0	16.6	14.0	12.3	18.3
13	6.00	17.0	20.0	13.5	14.0	n.d
14	13.0	13.0	14.0	21.0	16.0	13.0
15	11.0	13.5	18.5	18.0	n.d.	14.0
16	16.5	n.d.	20.0	13.4	15.0	15.0
17	18.5	n.d.	11.5	14.0	n.d.	n.d.

3.3 Diabetic Rat treated with Artemisia (27 day experiment):

This experiment was designed to try and established the beneficial effect, if any, of *Artemisia judaica* in diabetic rats.

Induction of diabetes caused the same rate of weight change as seen in the previous experiment (26.12±12.45g in control animals and -2.6±7.31g in diabetic animals) ($p<0.05$) . This was associated with the other characteristics of diabetes mellitus (i.e. polydipsia , polyuria (Figure 3.14) and hyperphagia. All animals showed clear glycosuria and significant signs of ketosis after induction of diabetes.

Replacement of the drinking water by a crude extract of *Artemisia judaica* on day 17 following induction of diabetes, led to a marked inhibition of all six rats to drink any extract solution and this led to dehydration see (Figure 3.11) and, thus, a marked decrease in body weight. This weight loss was so severe that rat 6 was sacrificed early. On day 19 the plant extract was replaced by water for 24 hours and then the dose of extract was gradually increased to allow body weight to stabilize while continuing treatment with *Artemisia* extract. Overall a small though non-significant reversal of the weight loss caused by diabetes was seen (before treatment 26.0±4.5g, after treatment with STZ -2.6±7.30g and after treatment with *Artemisia* extract (4.0±12.5g) (Figure 3.11). There was no improvement in food intake (before treatment with *Artemisia* ; 23.2±5.84g, after treatment with STZ ; 33.27± 9.52g and after treatment with *Artemisia* ; 41.86±7.1g) and, indeed, food intake increased further (Figure 3.12). Urine volume was also unchanged by *Artemisia* treatment (7.48±2.60ml in control animals, 191.97±39.41ml after treatment with STZ and 199.57±29.21ml with *Artemisia* extract (Figure 3.14) as was the fluid intake (Figure 3.13) and faeces weight (Table 3.4). Significant results were found for urine glucose and ketone levels. Glucose levels were reduced on the last 2 days of *Artemisia* treatment (Figure 3.15) often the urine tested negative for ketones in the animals treated with *Artemisia* extract (Table 3.3).

Figure 3.11 The effect of STZ-induced diabetes and *Artemisia judaica* extract treatment on body weight.

(1) = Treatment with STZ (65mg / kg) .

(2) = Start of treatment with *Artemisia* extract in a and b as.

o= rat (1, 4); • = rat (2, 5); ∇ = rat (3, 6)

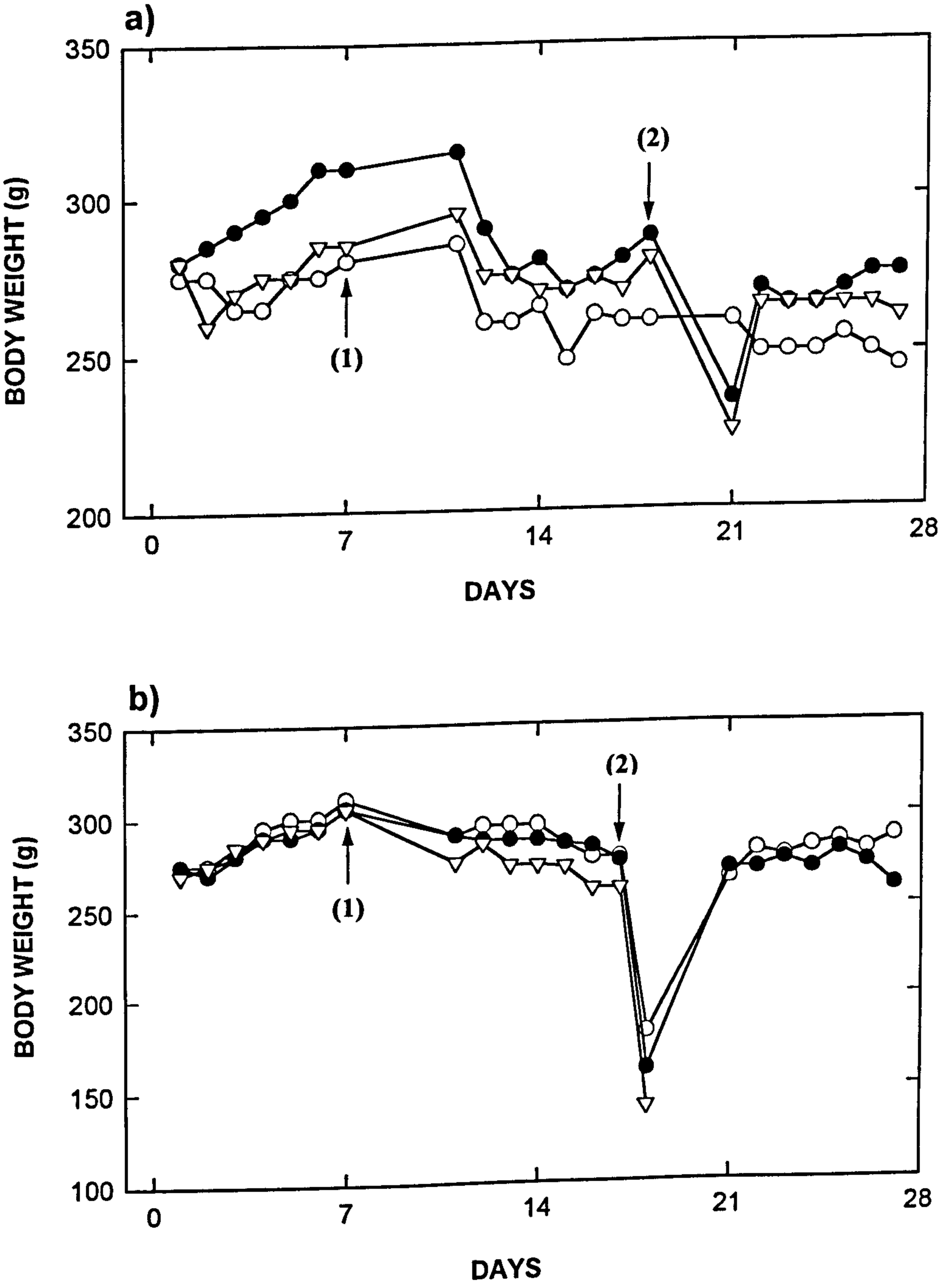
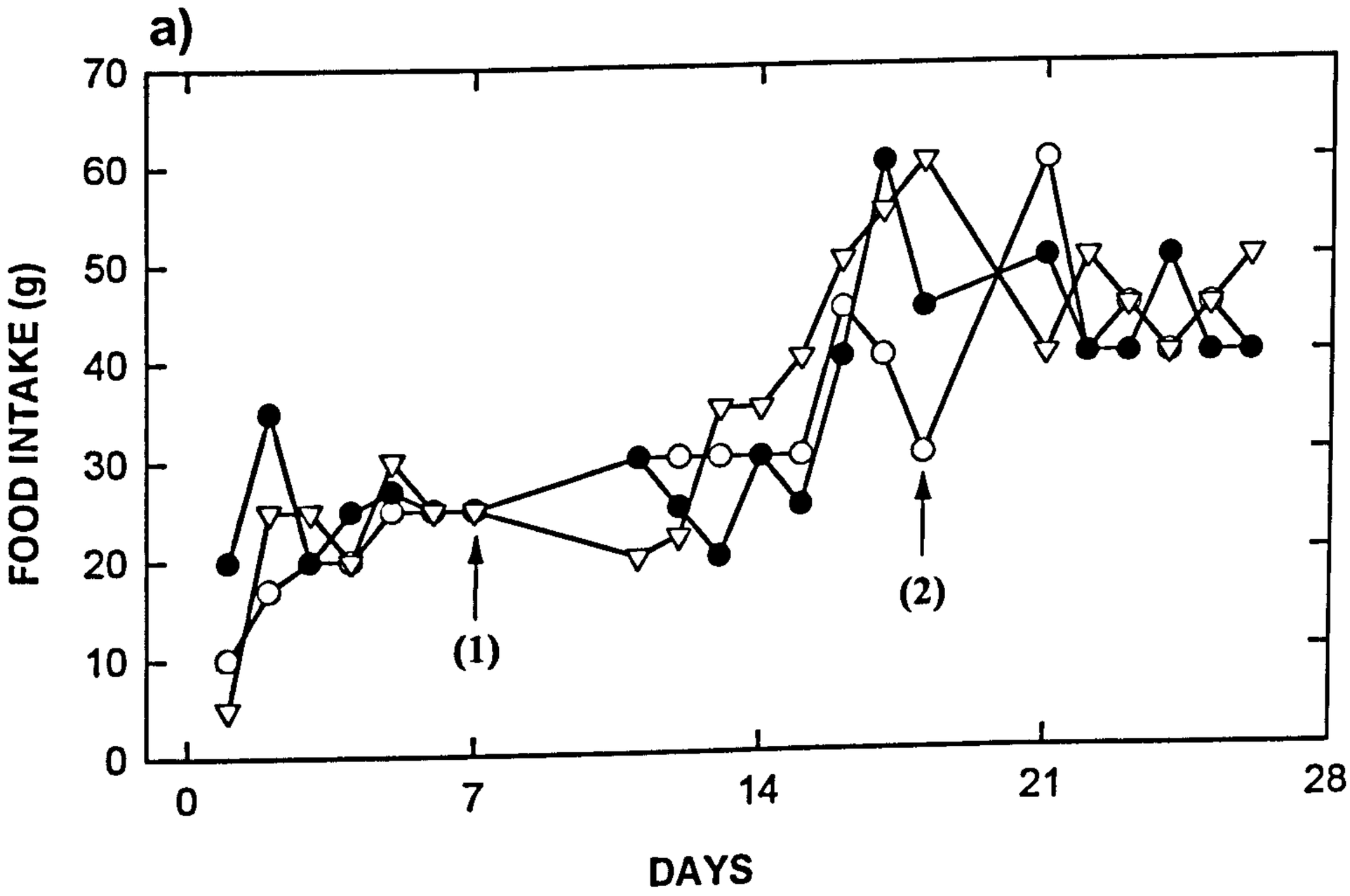

a)
350
300
250
200
BODY WEIGHT (g)
(1)
(2)
0
7
14
21
28
DAYS
b)
350
300
250
200
150
100
BODY WEIGHT (g)
(1)
(2)
0
7
14
21
28
DAYS

Figure 3.12 The effect of STZ-induced diabetes and *Artemisia judaica* treatment on food intake

(1) = Treatment with STZ (65mg / kg).

(2) = Start of treatment with *Artemisia* extract in a and b as.

o= rat (1, 4); • = rat (2, 5); ∇ = rat (3, 6)


a)
70
60
50
40
30
20
10
0
FOOD INTAKE (g)
0
7
14
21
28
(1)
(2)
DAYS

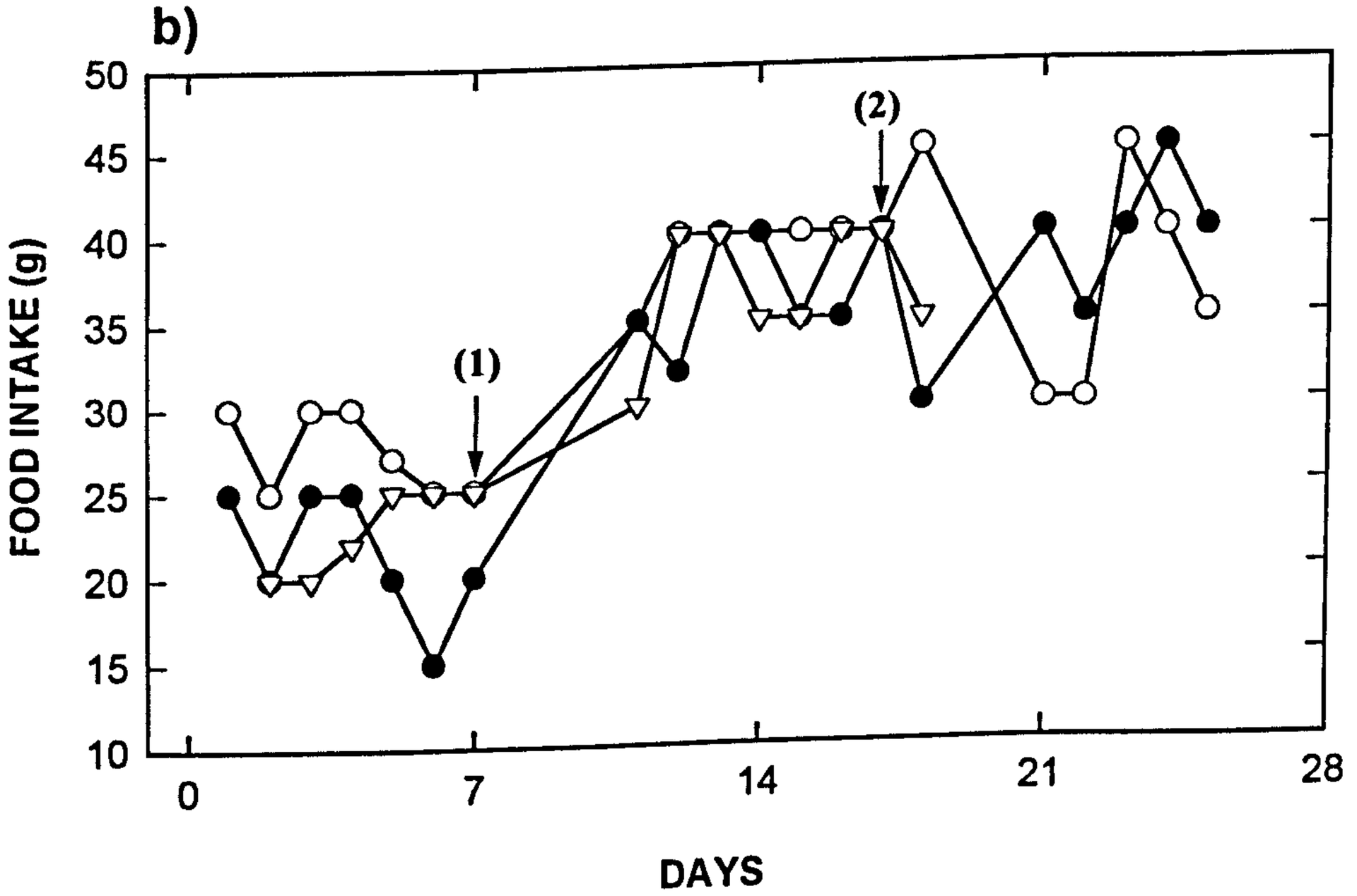

b)
50
45
40
35
30
25
20
15
10
FOOD INTAKE (g)
0
7
14
21
28
(1)
(2)
DAYS

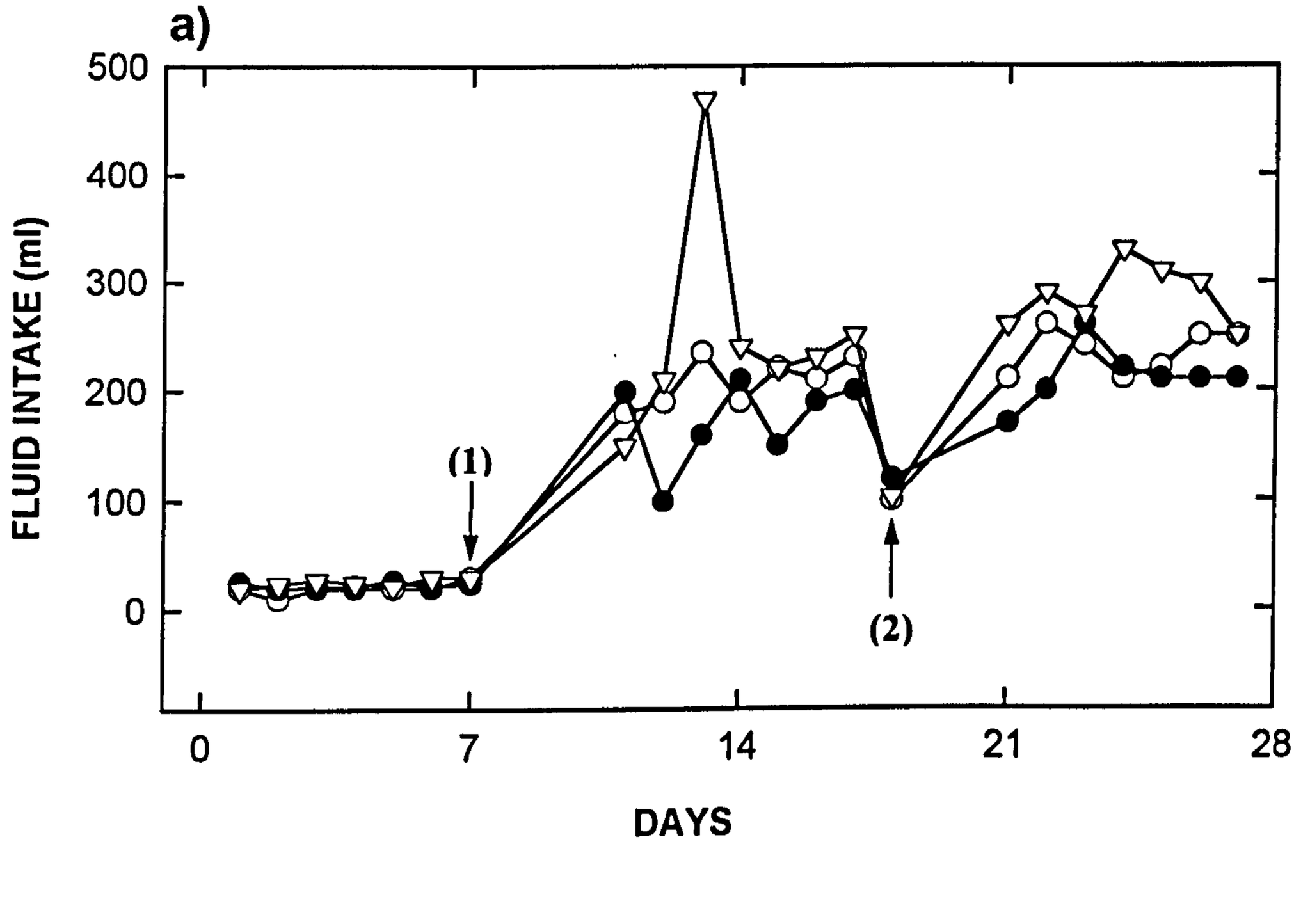
Figure 3.13 The effect of STZ-induced diabetes and *Artemisia judaica* treatment on fluid intake.

(1) = Treatment with STZ (65mg / kg).

(2) = Start of treatment with *Artemisia* extract in a and b as.

o= rat (1, 4); • = rat (2, 5); ∇ = rat (3, 6)

a)


500
400
300
200
100
0
FLUID INTAKE (ml)
(1)
(2)
0
7
14
21
28
DAYS

b)

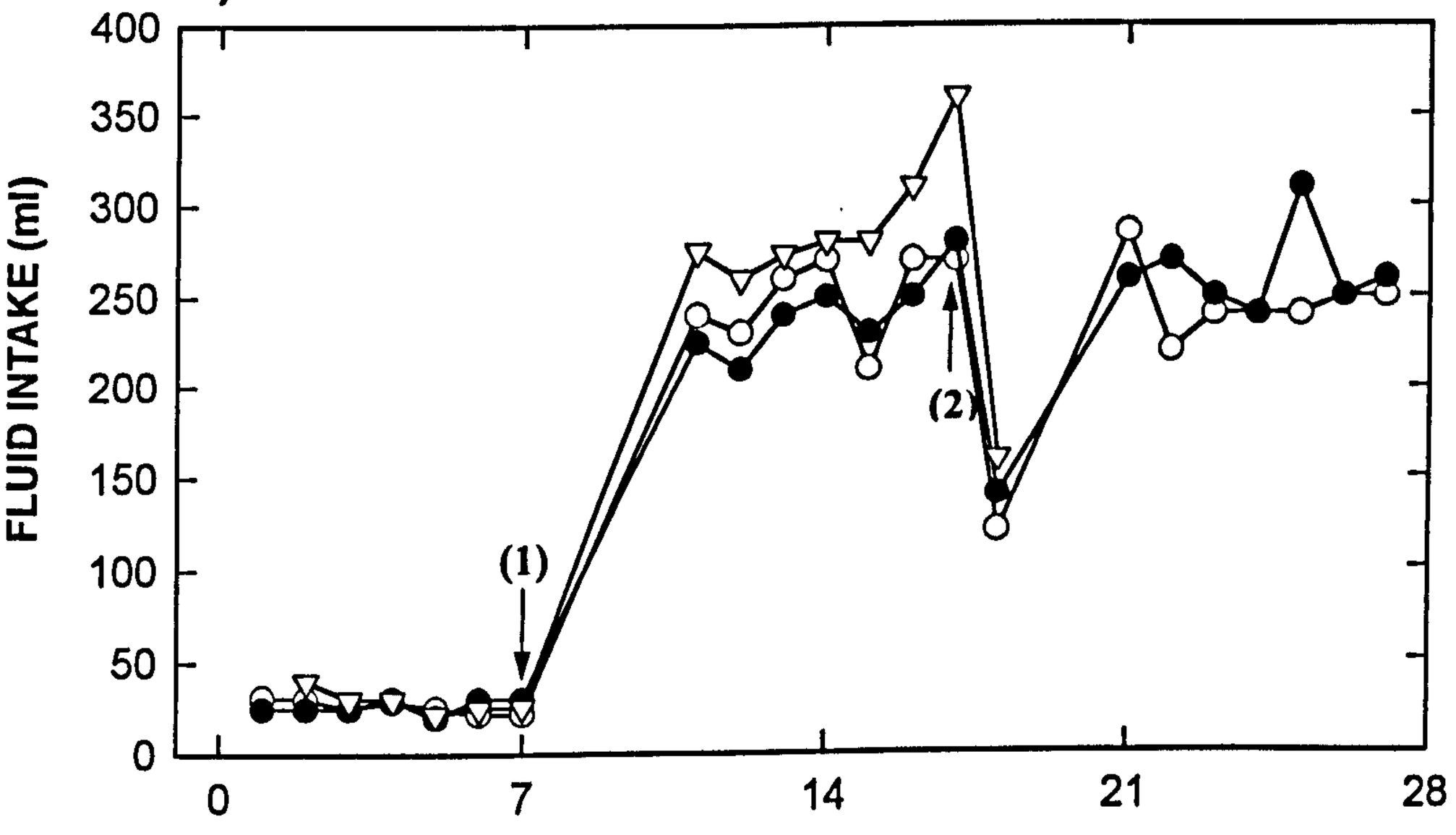

400
350
300
250
200
150
100
50
0
FLUID INTAKE (ml)
(1)
(2)
0
7
14
21
28
DAYS

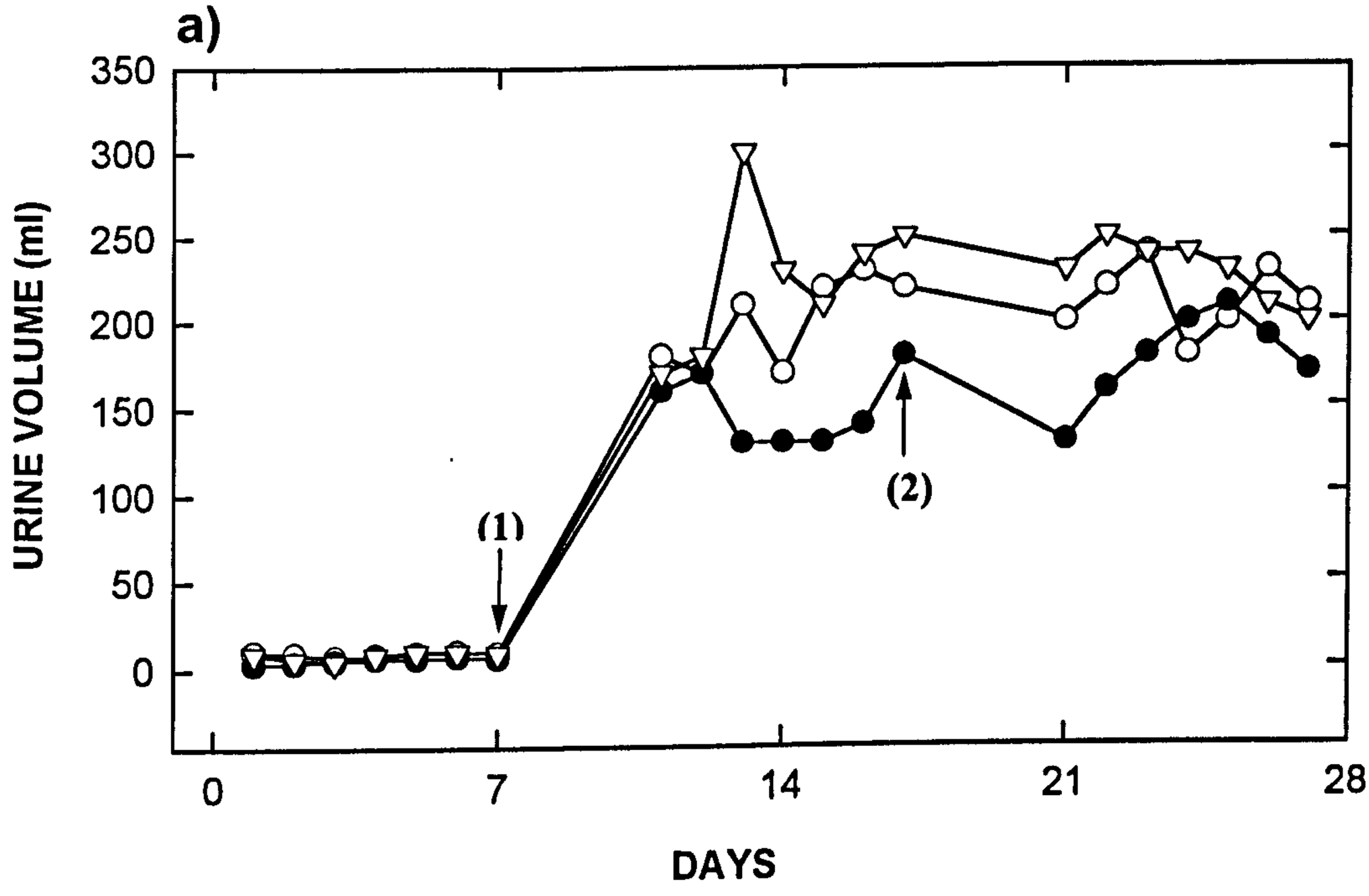
Figure 3.14 The effect of STZ-induced diabetes and *Artemisia judaica* extract treatment on urine volume.

(1) = Treatment with STZ (65mg / kg).

(2) = Start of treatment with *Artemisia* extract in a and b as.

o= rat (1, 4); • = rat (2, 5); ∇ = rat (3, 6)

a)


350
300
250
200
150
100
50
0
URINE VOLUME (ml)
(1)
(2)
0
7
14
21
28
DAYS

b)

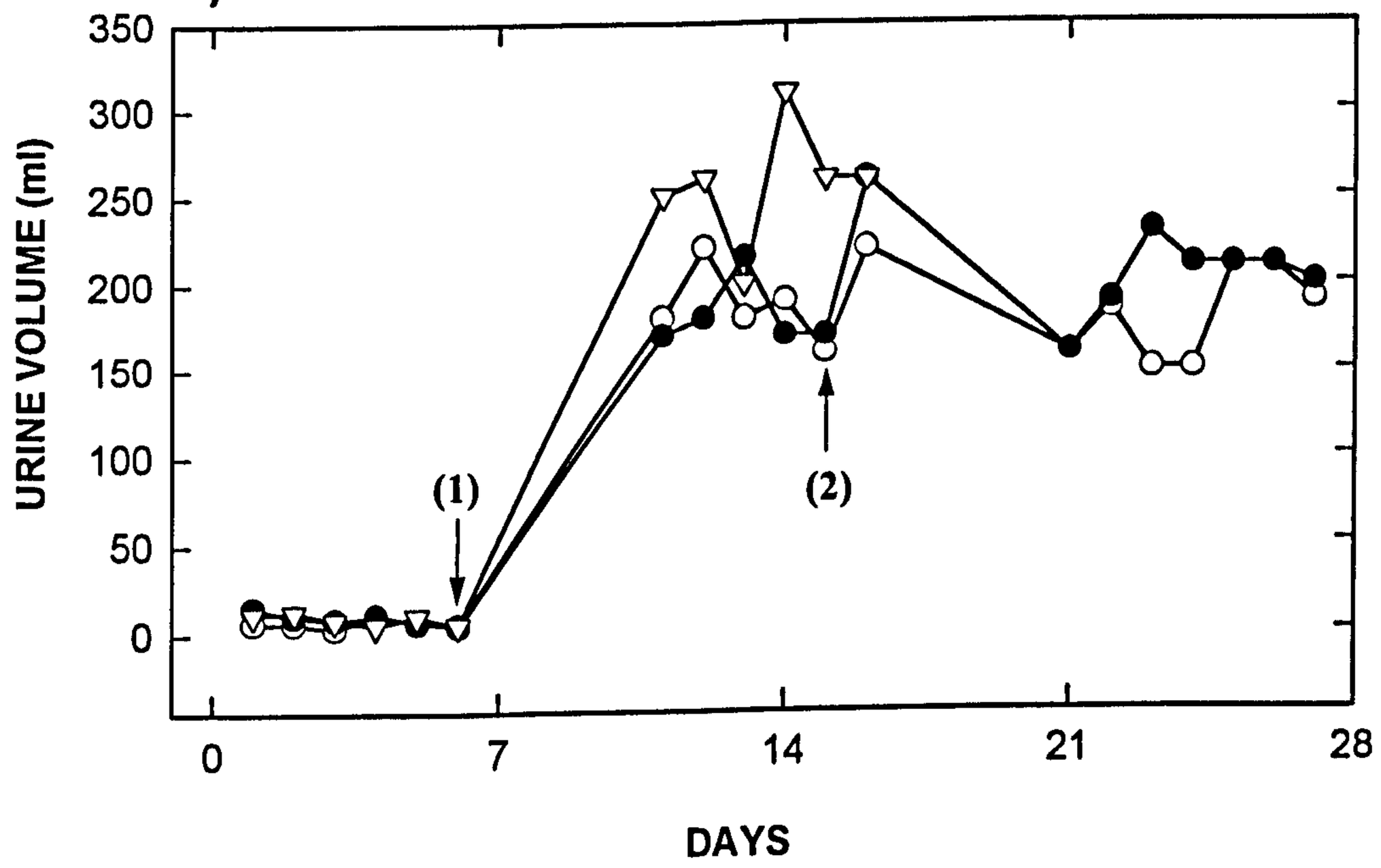

350
300
250
200
150
100
50
0
URINE VOLUME (ml)
(1)
(2)
0
7
14
21
28
DAYS

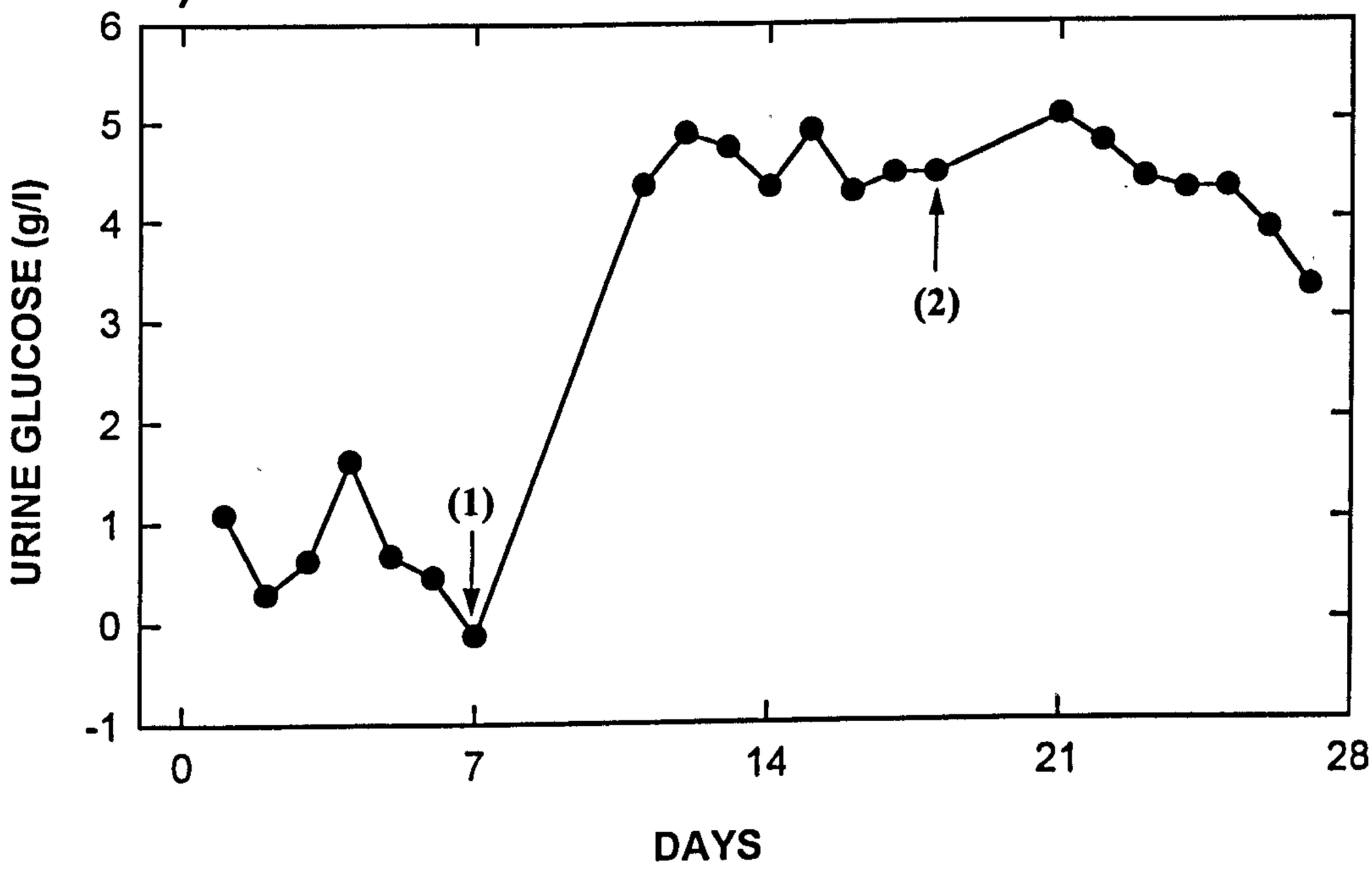
Figure 3.15 The effect of STZ-induced diabetes and *Artemisia judaica* extract treatment on urine glucose.

(1) = Treatment with STZ (65mg / kg).

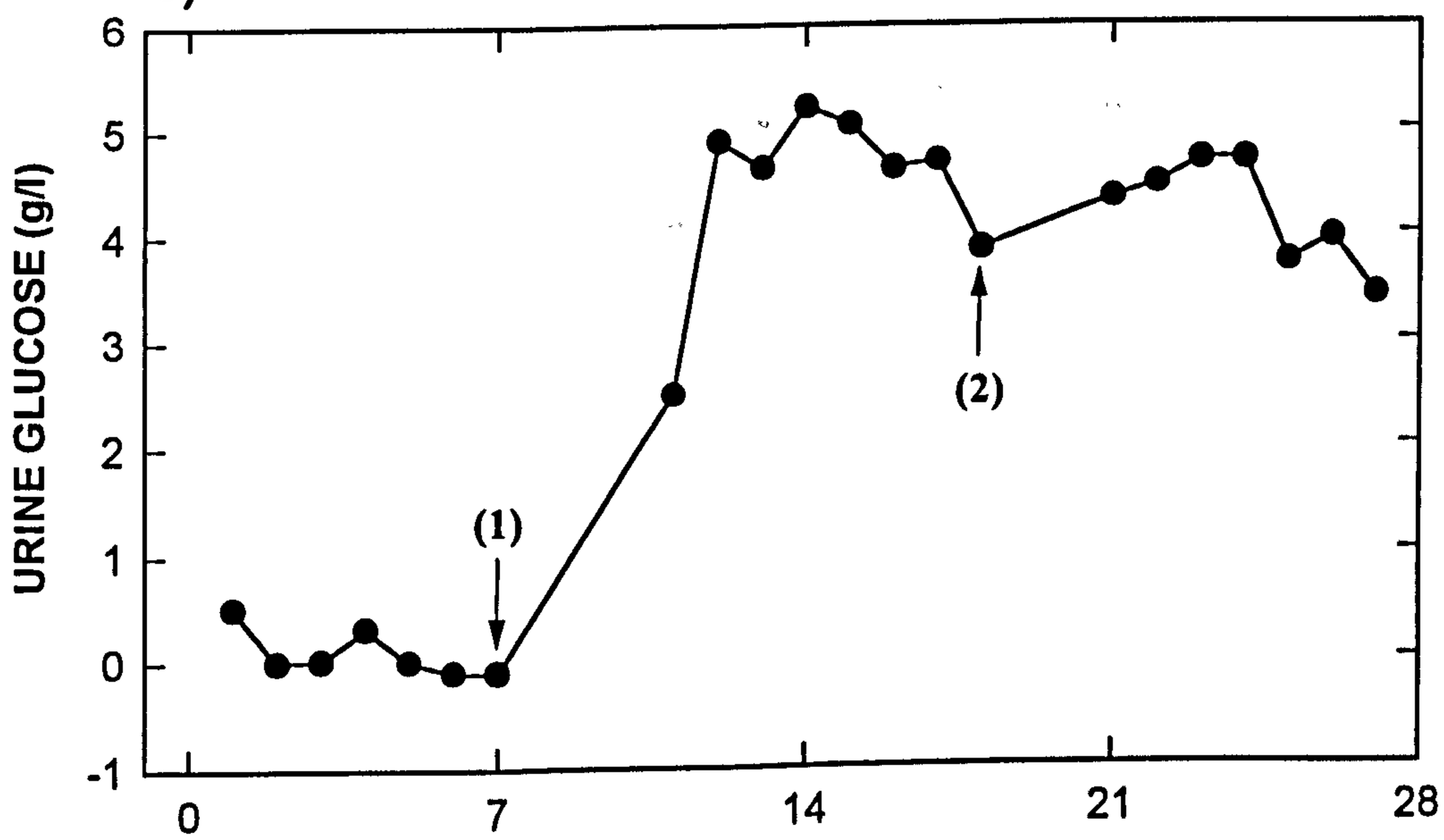
(2) = Start of treatment with *Artemisia* extract in a and b as.

b = rat 2; c = rat 3; d = rat 4 & e = rat 5.

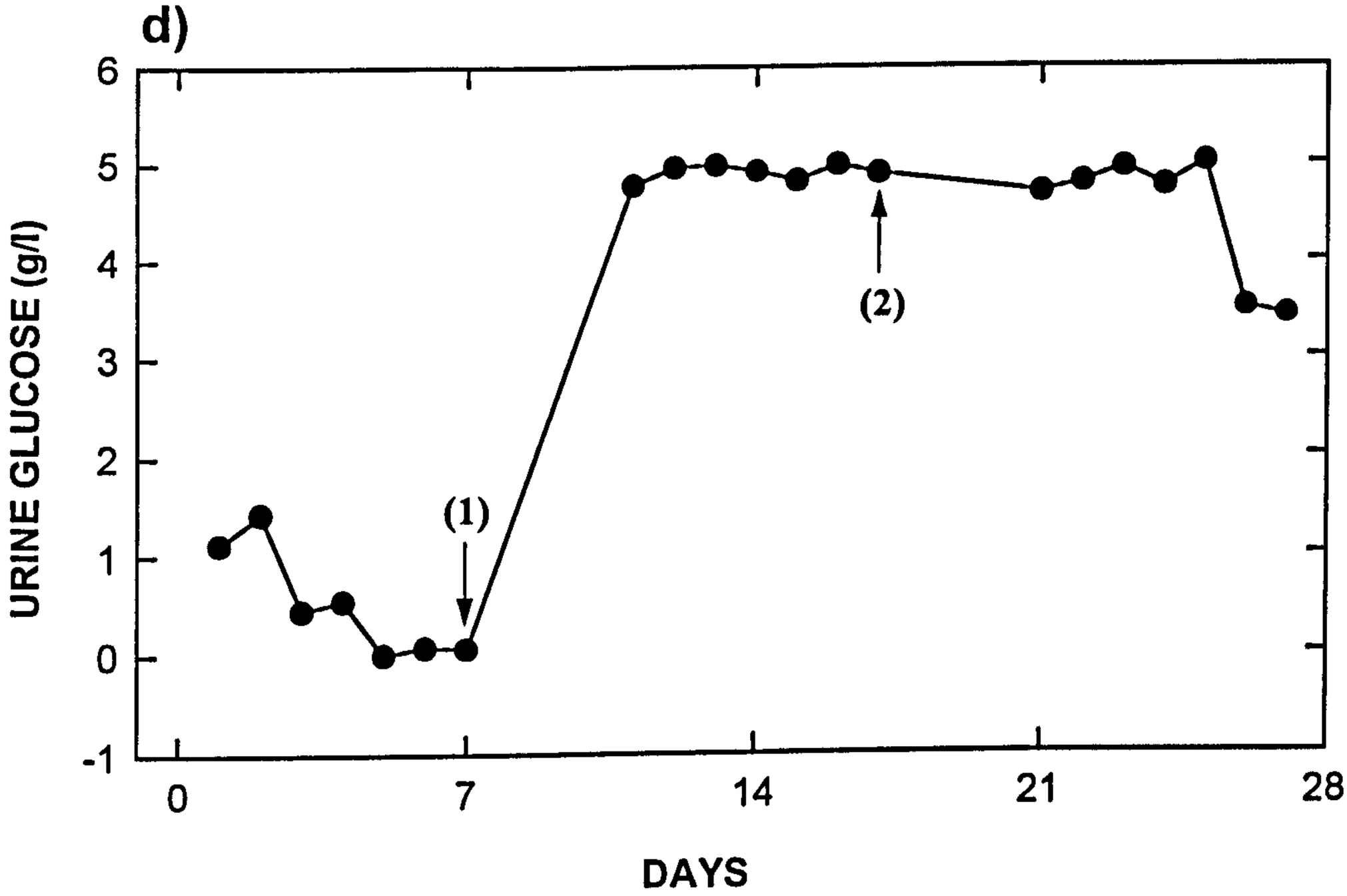
b)


URINE GLUCOSE (g/l)
6
5
4
3
2
1
0
-1
0
7
14
21
28
DAYS
(1)
(2)

c)


URINE GLUCOSE (g/l)
6
5
4
3
2
1
0
-1
0
7
14
21
28
DAYS
(1)
(2)

d)


URINE GLUCOSE (g/l)
6
5
4
3
2
1
0
-1
0
7
14
21
28
DAYS
(1)
(2)

e)

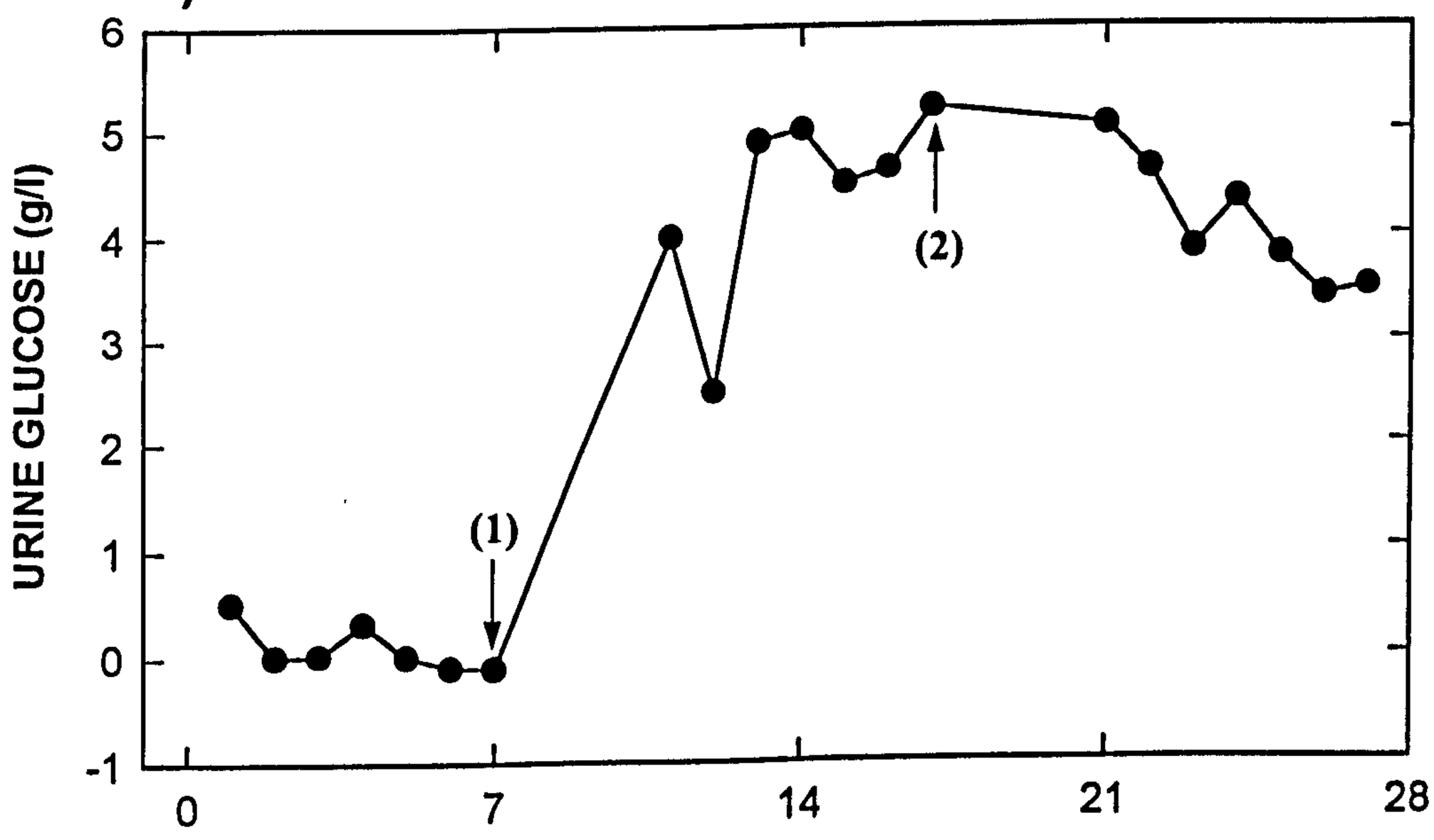

URINE GLUCOSE (g/l)
6
5
4
3
2
1
0
-1
0
7
14
21
28
DAYS
(1)
(2)

Table 3.3: Urine ketone of diabetic rats treated with *Artemisia judaica* (STZ was given on day 7 and treatment begun on day 17. & n.d = not determined)

Urine Ketone	DAYS	RAT1	RAT2	RAT3	RAT4	RAT5	RAT6
	1	-ve	-ve	-ve	-ve	-ve	-ve
	2	-ve	-ve	-ve	-ve	-ve	-ve
	3	-ve	-ve	-ve	-ve	-ve	-ve
	4	-ve	-ve	-ve	-ve	-ve	-ve
	5	-ve	-ve	-ve	-ve	-ve	-ve
	6	-ve	-ve	-ve	-ve	-ve	-ve
	7	-ve	-ve	-ve	-ve	-ve	-ve
	11	++	++	+	n.d.	+++	+++
	12	+++	++	+	+++	+++	+++
	13	+++	+++	++	++	+++	+++
	14	+++	+++	+++	+++	+++	+++
	15	+++	+++	+++	+++	+++	+++
	16	++	++	+++	+++	+++	+++
	17	+++	+++	++	+++	+++	+++
	21	+	++	++	++	++	n.d.
	22	++	+	+	-ve	+	n.d.
	23	-ve	+	+	-ve	++	n.d.
	24	-ve	-ve	-ve	++	-ve	n.d.
	25	+	-ve	-ve	-ve	-ve	n.d.
	26	+	-ve	-ve	-ve	+	n.d.

Table 3.4: Faeces (g) weight of diabetic rat treated with *Artemisia judaica* (STZ was given on day 7 and treatment begun on day 17. & n.d = not determined)

Faeces Weight(g	DAYS	RAT1	RAT2	RAT3	RAT4	RAT5
	1	3.30	6.31	4.91	8.50	4.91
	2	6.50	9.90	5.780	7.80	5.60
	3	7.50	11.4	11.1	6.20	7.40
	4	6.50	6.50	5.50	12.0	7.50
	5	9.00	9.50	11.0	7.50	5.90
	6	8.50	7.50	6.00	n.d	n.d
	7	8.50	7.50	6.00	n.d	n.d
	11	10.5	16.0	10.0	13.5	20.0
	12	15.0	5.50	7.50	14.0	10.5
	13	10.0	9.30	13.5	20.0	18.0
	14	15.0	10.4	12.5	17.0	14.0
	15	14.2	14.43	10.0	15.0	10.5
	16	18.5	16.5	17.0	16.0	13.5
	17	n.d	n.d	n.d	n.d.	n.d.
	21	n.d.	16.0	18.5	19.0	n.d.
	22	n.d.	11.5	14.0	15.1	n.d.
	23	n.d.	11.5	13.5	13.5	n.d.
	24	n.d.	16.5	15.5	16.5	n.d.
	25	n.d.	19.0	16.5	17.0	n.d.
	26	n.d.	16.0	24.4	14.0	n.d.

3.4 Discussion:

Before discussing the results obtained from the *in-vivo* experiments, a few general comments concerning the overall results need to be made. One problem experienced during this project, which is common to all *in-vivo* experimentation, is subject individuality which can possibly cause a wide range of variance between experimental groups and individual animals within an experimental group. We tried to minimise this problem by using each animal as its own control - i.e. the same animal was assessed before treatment, after induction of diabetes and during *Artemisia* treatment, as and where appropriate. One other biological variation has to be considered, however, and that is the plant. The site from which the plant was collected and the time of plant collection as well as the maturity of the plant at collection time can lead to variance and different in the results. Where possible a full set of experiments was performed with a single extraction of the plant material.

Another significant limitation on the project directly related to the *in-vivo* experiments was that the fluid intake in all diabetic animals was significantly reduced in the presence of *Artemisia* extract due to the bitter taste. This led to dehydration of the animals which caused a significant decrease in the body weight in 5 rats and, indeed, one of the rats (rat 6) was sacrificed due to excessive weight loss. This led to a modification of the experimental schedule as described above.

One of the first observations made early on in the experiments was that the animals suffered from stress upon entry into the metabolic cages. This was manifested as agitated behaviour, increased urine volume and glucose content (stress polyuria and glycosuria), reductions in food and fluid intake which increased the apparent weight loss in the rats during the first 24 hours. This problem was overcome by not taking measurements from the rats until they had been in the metabolic cages for 24 hours. This meant most animals had re-established a normal food and fluid consumption patterns by the start of the recording period.

The induction of diabetes with STZ was followed by a three day recovery period to reduce the chance that any experimental compounds were producing seemingly beneficial effects simply by acting as radical scavengers and preventing or reducing the effects of STZ. The diabetogenic effect of STZ seen in the present study is in agreement

with those who have previously reported a severe hyperglycaemia and glucosuria in rats (Bone, 1990, Salhanick *et al.*, 1983).

The initial investigation of *Artemisia judaica* extract in normal animals produced interesting results. Urine volume was found to increase substantially despite fluid intake remaining at a constant level. This would suggest that *Artemisia judaica* is itself acting as a mild diuretic. We were concerned about the apparent presence of glucose in the urine of normal animals following exposure to *Artemisia* extract in the first 6 rats. A possible explanation for this may be the presence of a substance in the *Artemisia* extract that reacts in the glucose assay but which is not re-absorbed through the kidney. Indeed, assay of the *Artemisia* extract itself revealed that there is a cross-reacting substance in the extract, the nature of which is unknown at present (giving an apparent glucose reading of 3.49 g/l). Some preliminary ideas about the contents of the extract are discussed later. The effect noted may be similar to an osmotic diuretic (e.g. mannitol).

The effect of *Artemisia* extract on the STZ diabetic animals was fairly small but included a decrease in glucose urine level in the last 2 days of treatment and a decrease in the presence of ketones from the urine within 72 hours of the extract first being administered. This reduced incidence and severity of ketosis would suggest that there is an improvement in the handling or catabolism of lipids, both of which would be beneficial in diabetes. There is, thus, some indication that a crude extract of *Artemisia judaica* can alleviate some of the symptoms of diabetes mellitus. The overall lack of dramatic effect of treatment with *Artemisia* extract in the *in-vivo* experiments could be due to an inadequate dose of *Artemisia* or to the relatively short treatment period. These experiments will need to be repeated with larger doses of *Artemisia* extract (if the animals will drink it) and / or longer periods of treatment.

4. Post *In-Vivo* liver parameters

At the end of the *in-vivo* experiments described above, the animals were sacrificed, the liver removed and washed and microsomes prepared as described in Materials & Methods. The following results were obtained:

4.1 Liver weight as percentage (%) of body weight

The effect of streptozotocin-diabetes and treatment with *Artemisia* extract on liver weight (as % of body weight) are shown in Figure 4.1. The administration of streptozotocin caused a significant decrease in liver weight (expressed as % of body weight) as compared to normal livers ($p<0.05$). An extract of *Artemisia* given to streptozotocin-induced diabetic rats caused the liver weights to return to normal and, in some instances, above normal. The same observation was seen when *Artemisia* extract was given to normal rats.

4.2 Liver protein content:

The effect of streptozotocin on hepatic microsomal protein content in normal rats and the effects of *Artemisia* extract in diabetic rats is shown in Figure 4.2. The administration of streptozotocin induced a significant increase in protein content ($p<0.05$) whereas the extract of *Artemisia* given to normal rats caused a decrease in protein content. To make sure this was an effect of *Artemisia* extract *in-vivo* and not something interfering with the protein assay, the extract was added to microsomes *in vitro* and caused no change in protein content.

4.3 Aniline 4-hydroxylase activity:

The effect of streptozotocin-induced diabetes and treatment with *Artemisia* extract on aniline 4-hydroxylase activity (expressed as nmol product/min/mg protein) is shown in Figure 4.3. The administration of streptozotocin caused a significant increase ($p<0.01$) in the enzyme activity. This increase was partially reversed by the administration of *Artemisia* extract to the diabetic rats but there was no significant effect on the enzyme

Figure 4.1 The effect of diabetes and *Artemisia* extract treatment on liver weight as percentage (%) of body weight.

Control = untreated; NOR + ART(*VIVO*) = normal rat treated with *Artemisia judaica* in-*vivo*; DIA + ART(*VIVO*) = diabetic rat treated with *Artemisia judaica* in-*vivo*, * = p< 0.05 compared to control

Results expressed as mean ± s.d.(for normal n = 9 and diabetic and diabetic treated with *Artemisia judaica* n = 6)

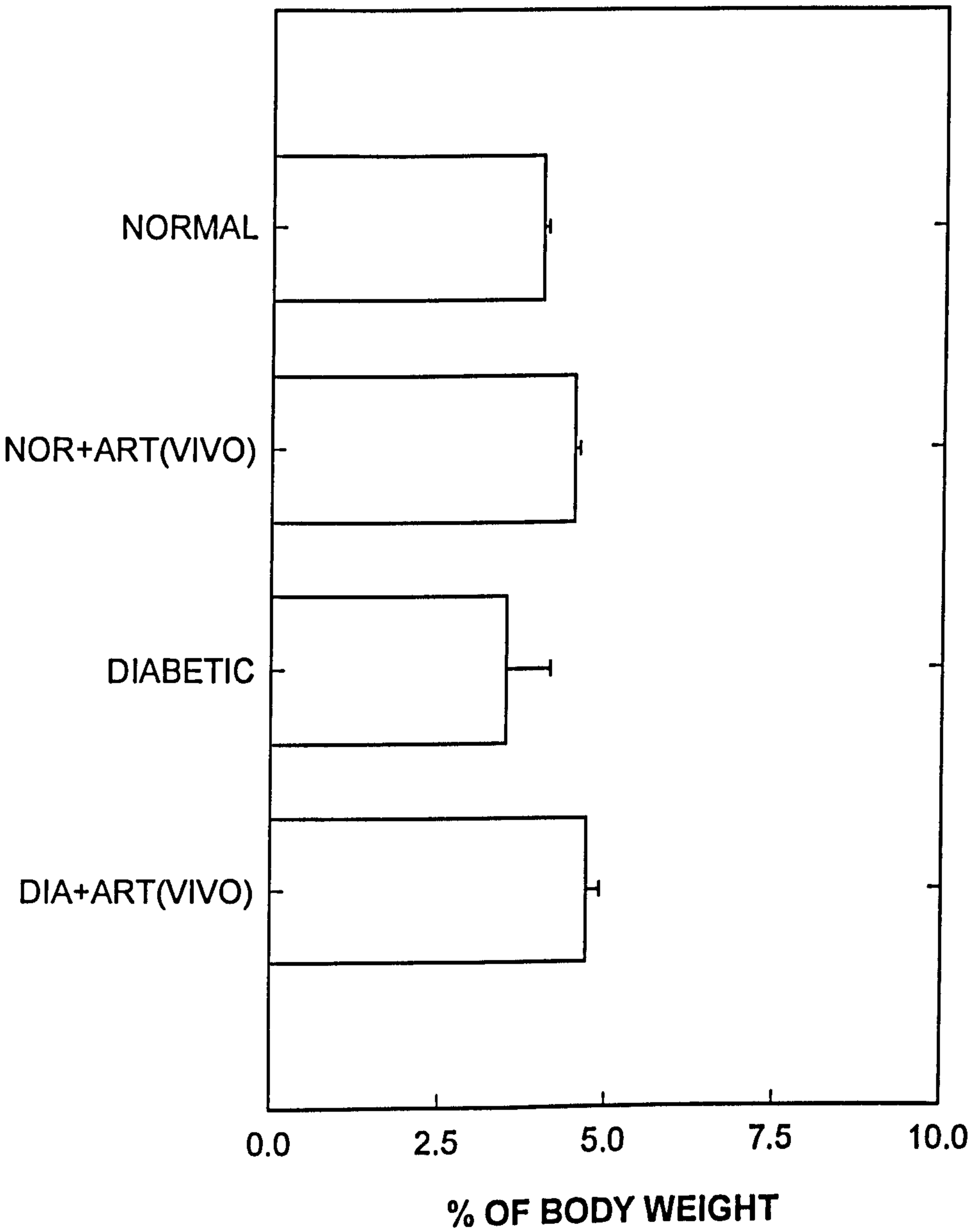

NORMAL
NOR+ART(VIVO)
DIABETIC
DIA+ART(VIVO)
0.0
2.5
5.0
7.5
10.0
% OF BODY WEIGHT

Figure 4.2 The effect of diabetes and *Artemisia* extract treatment on liver protein content.

Control = untreated; NOR + ART.(*VITRO*) = normal rat treated with *Artemisia judaica* in-*vitro*; NOR + ART(*VIVO*) = normal rat treated with *Artemisia judaica* in-*vivo*; DIA + ART(*VIVO*) = diabetic rat treated with *Artemisia judaica* in-*vivo*, * = $p < 0.05$ compared to control.

Results expressed as mean ± s.d.(for normal n = 9 and diabetic and diabetic treated with *Artemisia judaica* n = 6).

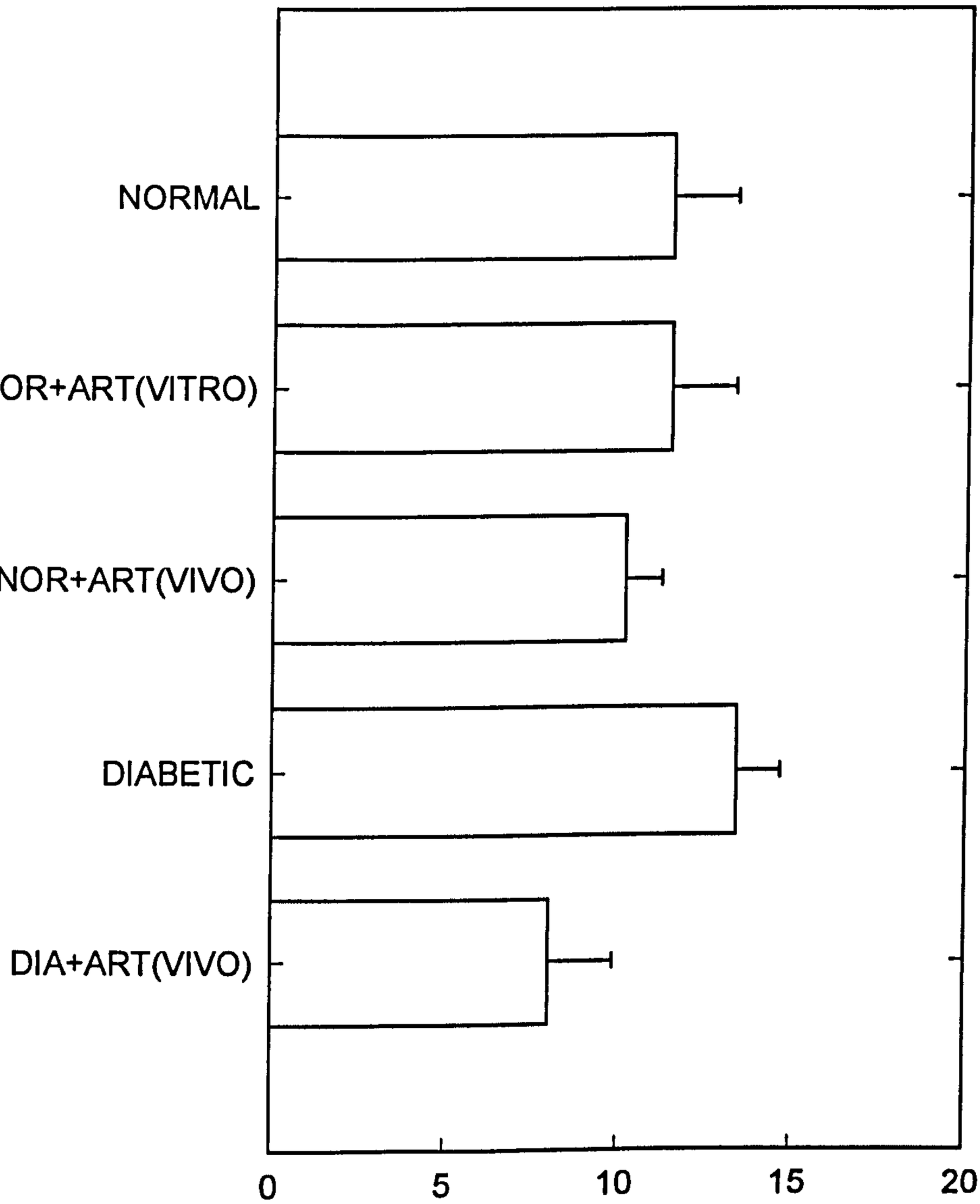
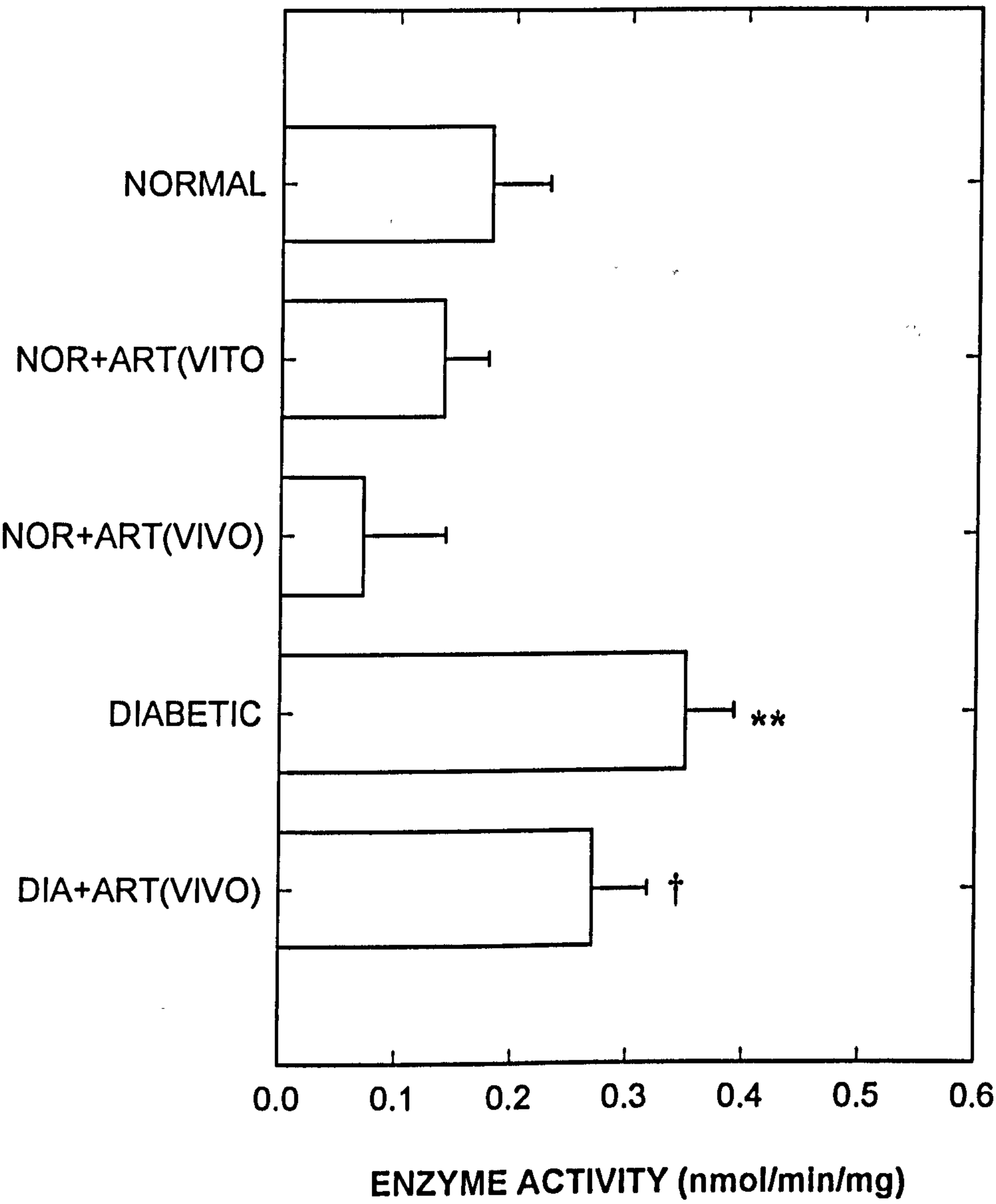

NORMAL
NOR+ART(VITRO)
NOR+ART(VIVO)
DIABETIC
DIA+ART(VIVO)
0
5
10
15
20
PROTEIN CONTENT (nmol/min/mg prot.)

Figure 4.3 The effect of diabetes and *Artemisia* extract treatment on aniline 4-hydroxylase.

Control = untreated; NOR + ART.(*VITRO*) = normal rat treated with *Artemisia judaica* in-*vitro*; NOR + ART(*VIVO*) = normal rat treated with *Artemisia judaica* in-*vivo*; DIA + ART(*VIVO*) = diabetic rat treated with *Artemisia judaica* in-*vivo*, ** = $p < 0.01$ compared to control rat & † = $p<0.05$ compared to diabetic rat.

Results expressed as mean ± s.d.(for normal n = 9 and diabetic and diabetic treated with *Artemisia judaica* n = 6).


NORMAL
NOR+ART(VITO
NOR+ART(VIVO)
DIABETIC
**
DIA+ART(VIVO)
†
0.0
0.1
0.2
0.3
0.4
0.5
0.6
ENZYME ACTIVITY (nmol/min/mg)

activity when *Artemisia* was added directly to normal microsomes (*in-vitro*). Normal rats treated with *Artemisia* extract showed little decrease in the enzyme activity.

4.4 Aminopyrine N-demethylase activity:

The effect of streptozotocin-induced diabetes and *Artemisia* extract treatment of normal and diabetic rats on aminopyrine *N*-demethylase activity is shown in Figure 4.4. The administration of streptozotocin caused a significant decrease in enzyme activity ($p<0.01$). This decrease was reversed by the administration of *Artemisia* extract to diabetic rats. The administration of *Artemisia* to normal rats showed no significant effect on the enzyme activity where as the addition of *Artemisia* extract directly to normal microsomes gave a significant increase in enzyme activity ($p<0.05$).

4.5 Liver steroid metabolism :

The effects of streptozotocin-induced diabetes and treatment with *Artemisia* extract on the five enzymes which metabolize the steroid androst-4-ene-3,17-dione are shown in Figures 4.5-4.9. The administration of streptozotocin induced a decrease in all of the enzyme activities except the 7α-hydroxylase, but when *Artemisia* extract was given to diabetic rats a significant increase in all enzyme activities was seen, especially the 7α- and 6β-hydroxylases ($p<0.001$) and the 5α-reductase ($p<0.05$). The addition of *Artemisia* extract directly to normal microsomes caused a significant change in all enzyme activities except the 16α-hydroxylase whereas the administration of *Artemisia* extract to normal rats caused a significant decrease in 17-oxosteroid oxidoreductase and 16α-hydroxylase activities but no significant change in 7α-hydroxylase and 5α-reductase activities.

Figure 4.4 The effect of diabetes and *Artemisia* extract treatment on aminopyrine N-demethylase activity.

Control = untreated; NOR + ART.(*VITRO*) = normal rat treated with *Artemisia judaica* in-*vitro*; NOR + ART(*VIVO*) = normal rat treated with *Artemisia judaica* in-*vivo*; DIA + ART(*VIVO*) = diabetic rat treated with *Artemisia judaica* in-*vivo*, * = $p<0.05$, * * = $p< 0.01$ compared to control rat & ††† = $p<0.05$ compared to diabetic rat.

Results expressed as mean ± s.d.(for normal n = 9 and diabetic and diabetic treated with *Artemisia judaica* n = 6).

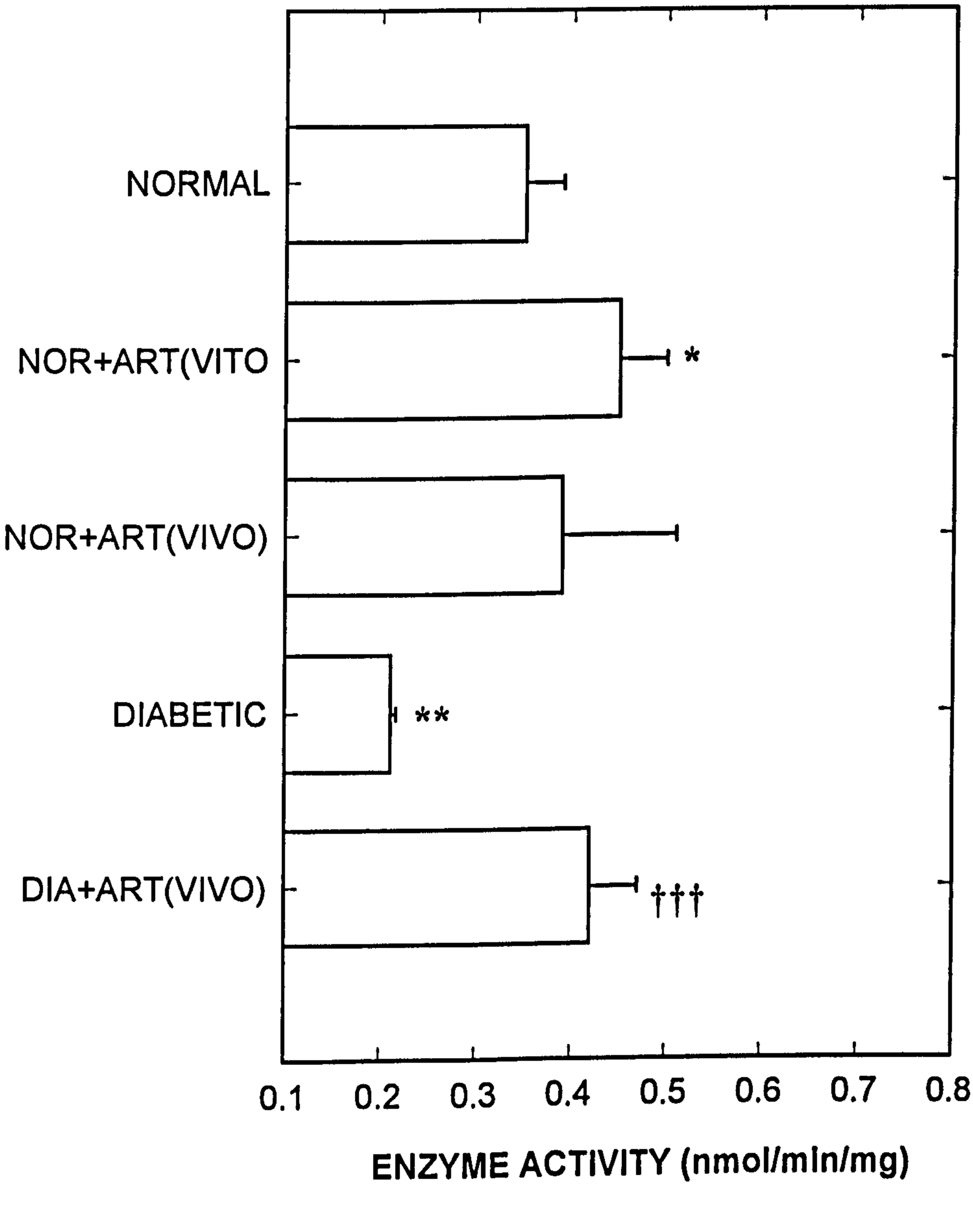

NORMAL
NOR+ART(VITO
*
NOR+ART(VIVO)
DIABETIC
**
DIA+ART(VIVO)
†††
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
ENZYME ACTIVITY (nmol/mIn/mg)

Figure 4.5 The effect of diabetes and *Artemisia* extract treatment on liver steroid metabolism (7α-hydroxylation of androst-4-ene-3,17-dione) by liver microsomes.

Control = untreated; NOR + ART.(*VITRO*) = normal rat treated with *Artemisia judaica* in-*vitro*; NOR + ART(*VIVO*) = normal rat treated with *Artemisia judaica* in-*vivo*; DIA + ART(*VIVO*) =diabetic rat treated with *Artemisia judaica* in-*vivo*. ** = p< 0.01, *** = p<0.001 compared to control rat & ††† = p<0.001 compared to diabetic rat.

Results expressed as mean ± s.d.(for normal n = 9 and diabetic and diabetic treated with *Artemisia judaica* n = 6).

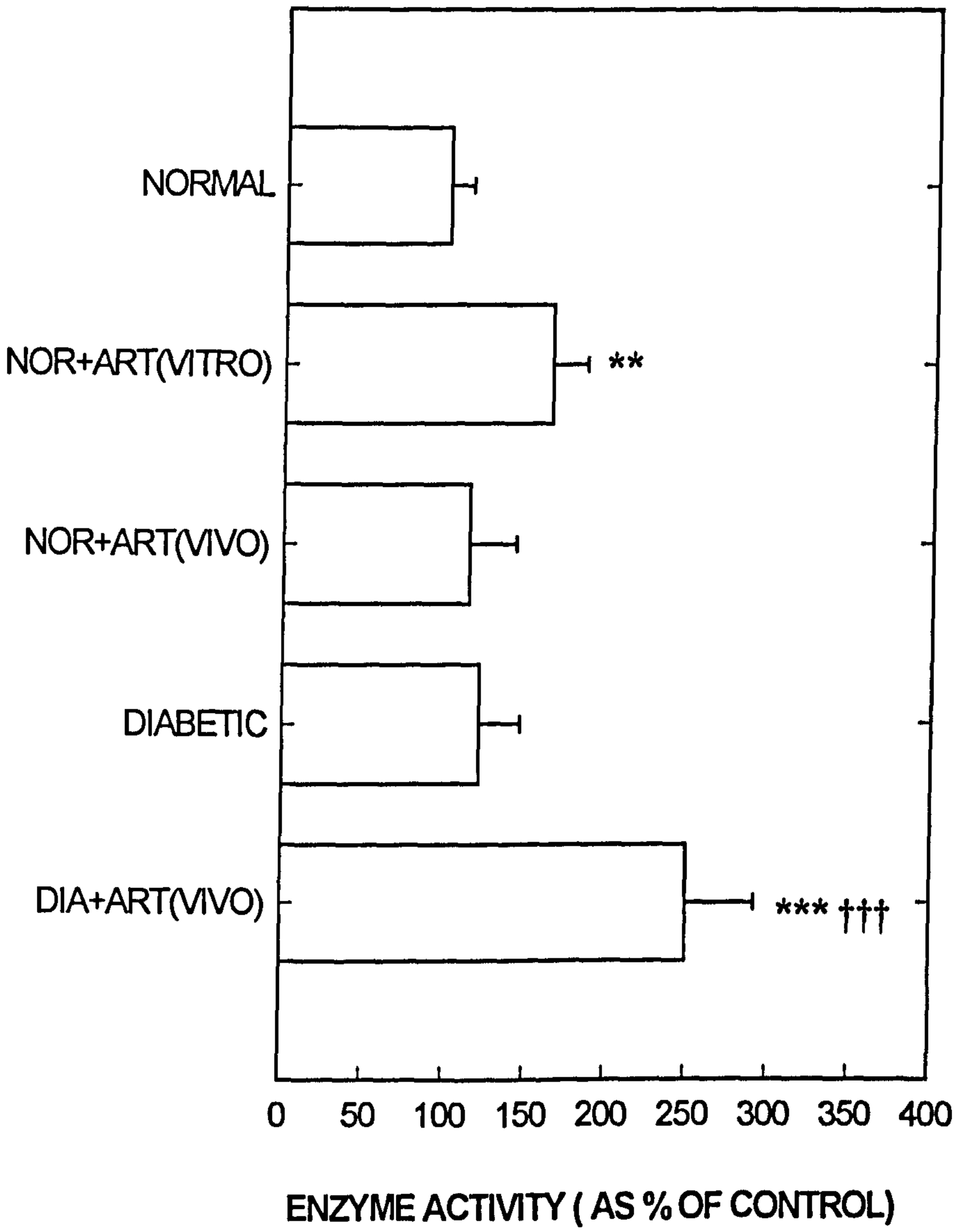

NORMAL
NOR+ART(VITRO)
**
NOR+ART(VIVO)
DIABETIC
DIA+ART(VIVO)
*** †††
0
50
100
150
200
250
300
350
400
ENZYME ACTIVITY (AS % OF CONTROL)

Figure 4.6 The effect of diabetes and *Artemisia* extract treatment on liver steroid metabolism (16β-hydroxylation of androst-4-ene-3,17-dione) by liver microsomes.

Control = untreated; NOR + ART.(*VITRO*) = normal rat treated with *Artemisia judaica* in-*vitro*; NOR + ART(*VIVO*) = normal rat treated with *Artemisia judaica* in-*vivo*; DIA + ART(*VIVO*) = diabetic rat treated with *Artemisia judaica* in-*vivo*, * = $p < 0.05$ compared to control.

Results expressed as mean ± s.d.(for normal n = 9 and diabetic and diabetic treated with *Artemisia judaica* n = 6).

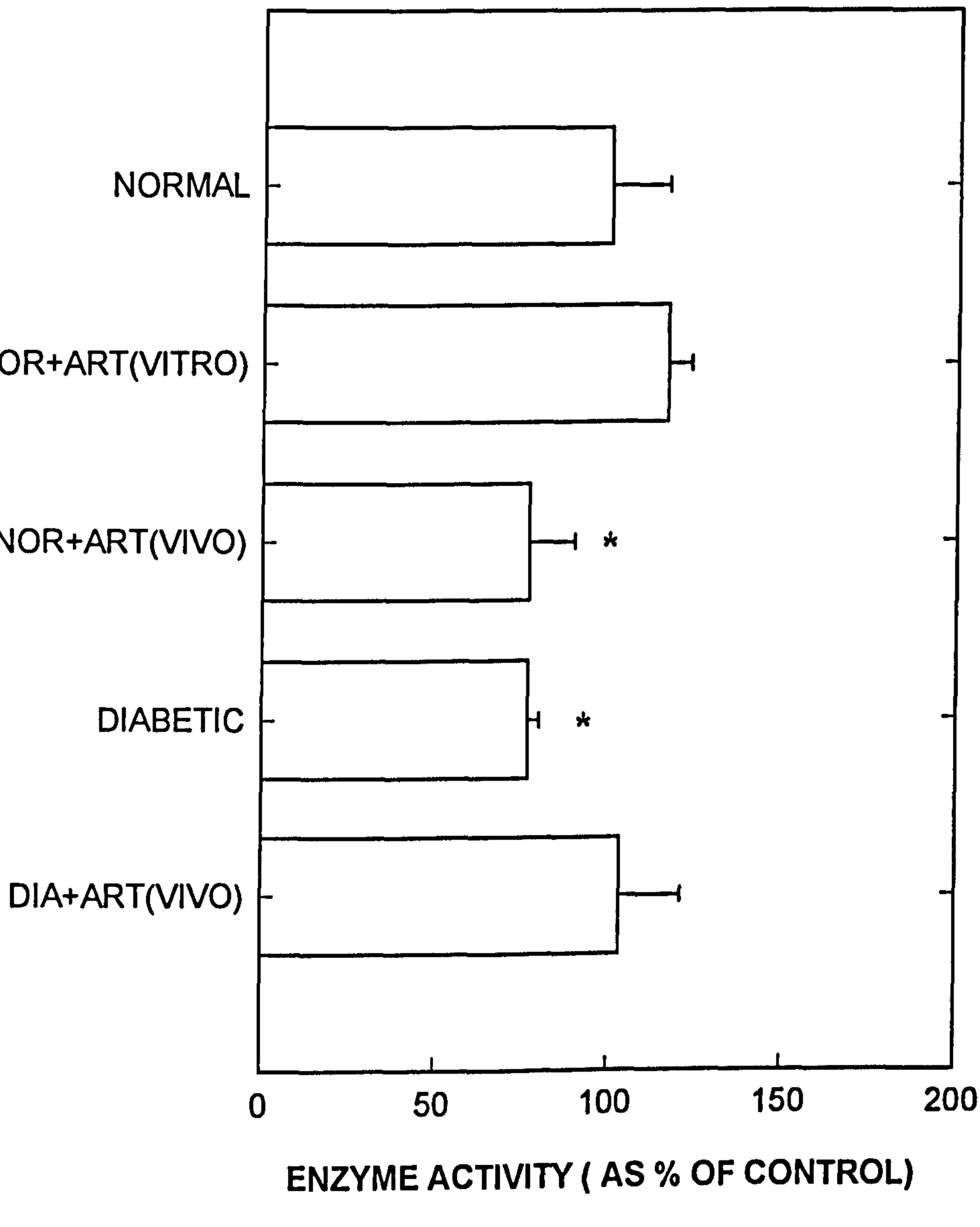

NORMAL
NOR+ART(VITRO)
NOR+ART(VIVO)
DIABETIC
DIA+ART(VIVO)
*
*
0
50
100
150
200
ENZYME ACTIVITY (AS % OF CONTROL)

Figure 4.7 The effect of diabetes and *Artemisia* extract treatment on (6β-hydroxylation of androst-4-ene-3,17-dione by rat liver microsomes.

Control = untreated; NOR + ART.(*VITRO*) = normal rat treated with *Artemisia judaica* in-*vitro*; NOR + ART(*VIVO*) = normal rat treated with *Artemisia judaica* in-*vivo*; DIA + ART(*VIVO*) = diabetic rat treated with *Artemisia judaica* in-*vivo*, * = p<o.o5, ** = p< 0.01 compared to control rat & ††† = p<0.001 compared to diabetic rat.

Results expressed as mean ± s.d.(for normal n = 9 and diabetic and diabetic treated with *Artemisia judaica* n = 6).

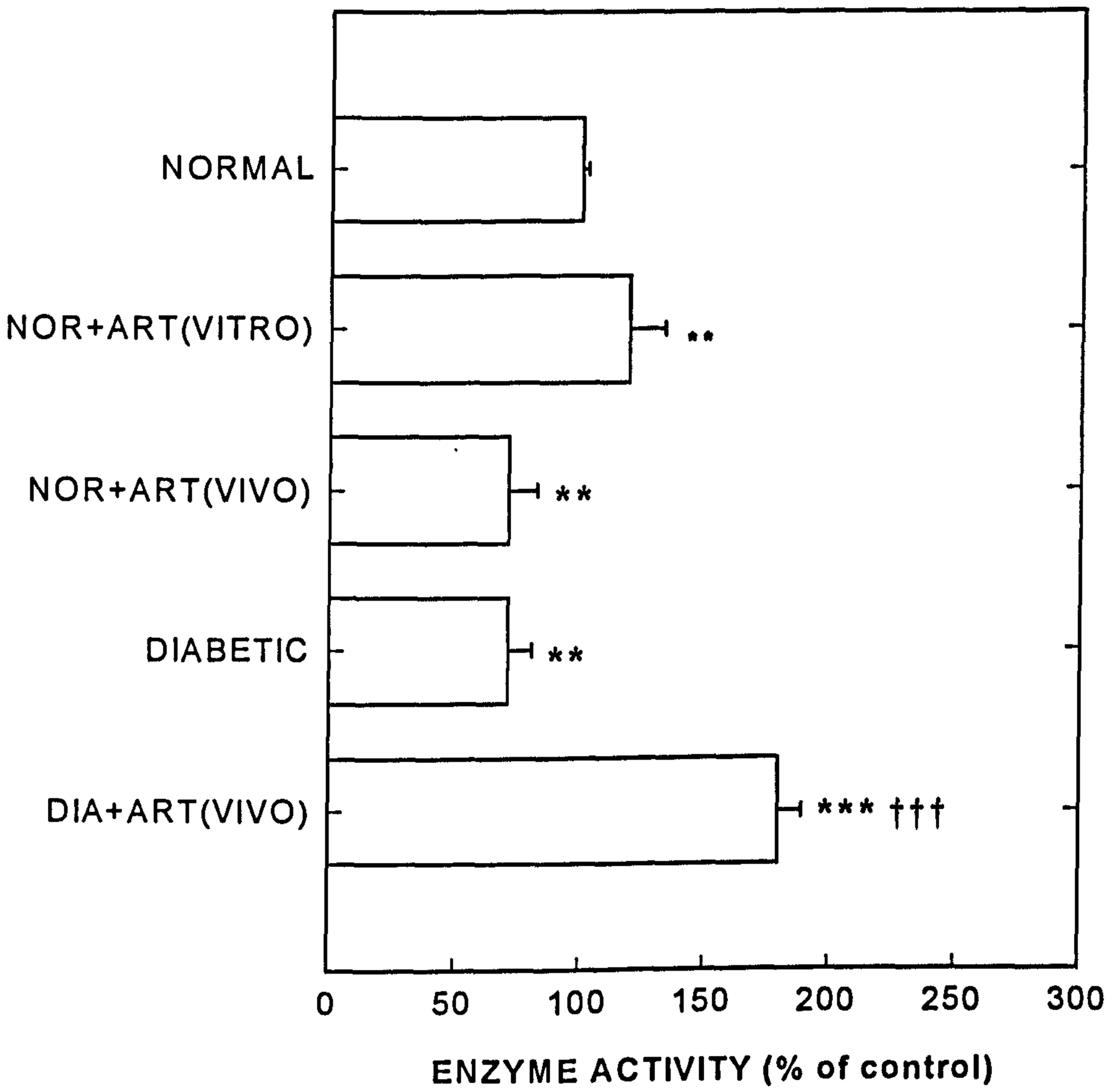

NORMAL
NOR+ART(VITRO)
**
NOR+ART(VIVO)
**
DIABETIC
**
DIA+ART(VIVO)
*** †††
0
50
100
150
200
250
300
ENZYME ACTIVITY (% of control)

Figure 4.8 The effect of diabetes and *Artemisia* extract treatment on 17α(β)-hydroxysteroid dehydrogenase of androst-4-ene3,17-dione by rat liver microsomes.

Control = untreated; NOR + ART.(*VITRO*) = normal rat treated with *Artemisia judaica* in-*vitro*; NOR + ART(*VIVO*) = normal rat treated with *Artemisia judaica* in-*vivo*; DIA + ART(*VIVO*) = diabetic rat treated with *Artemisia judaica* in-*vivo*, * = p<o.o5, ** = p< 0.01 compared to control rat & ††† = p<0.001 compared to diabetic rat...

Results expressed as mean ± s.d.(for normal n = 9 and diabetic and diabetic treated with *Artemisia judaica* n = 6).

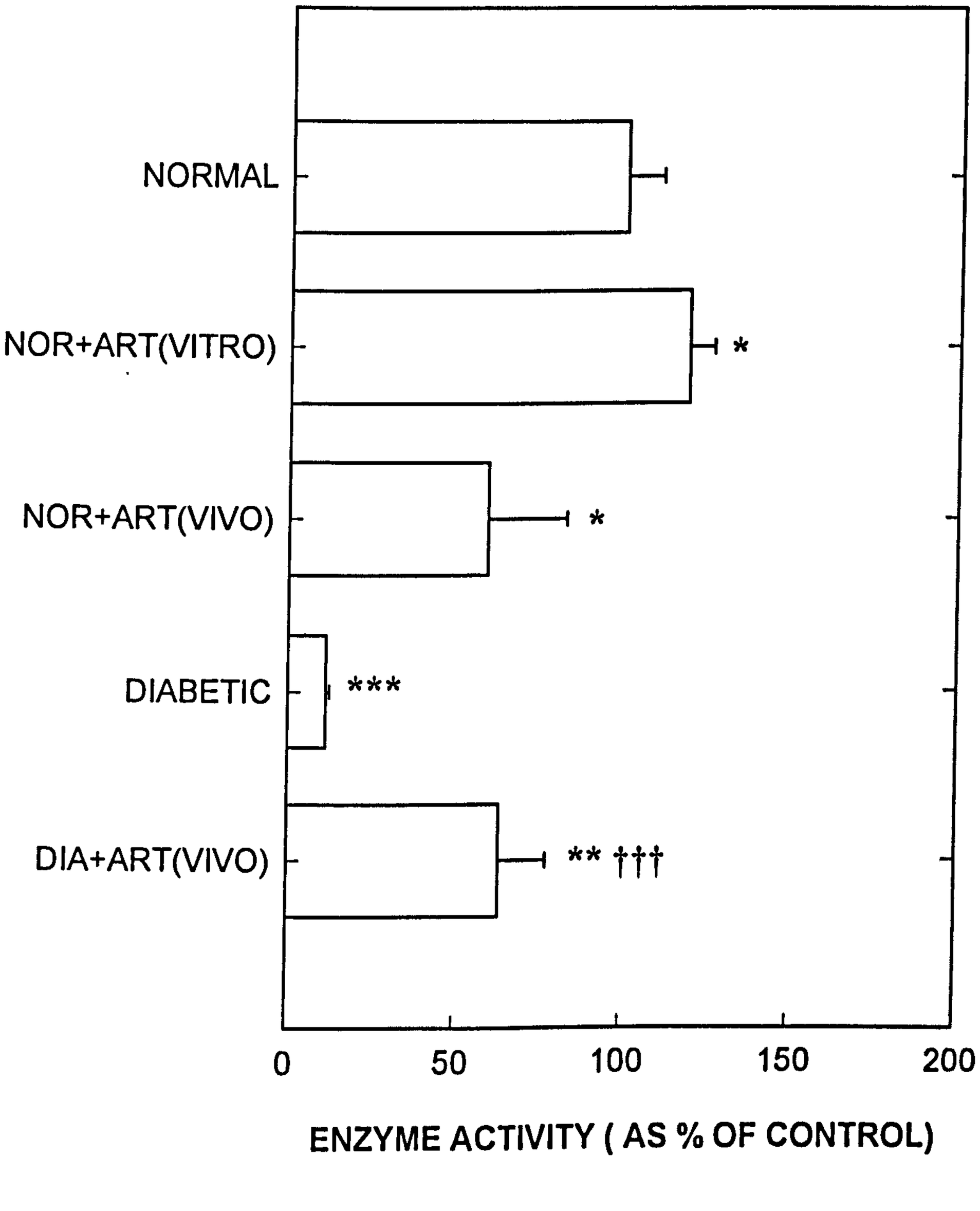
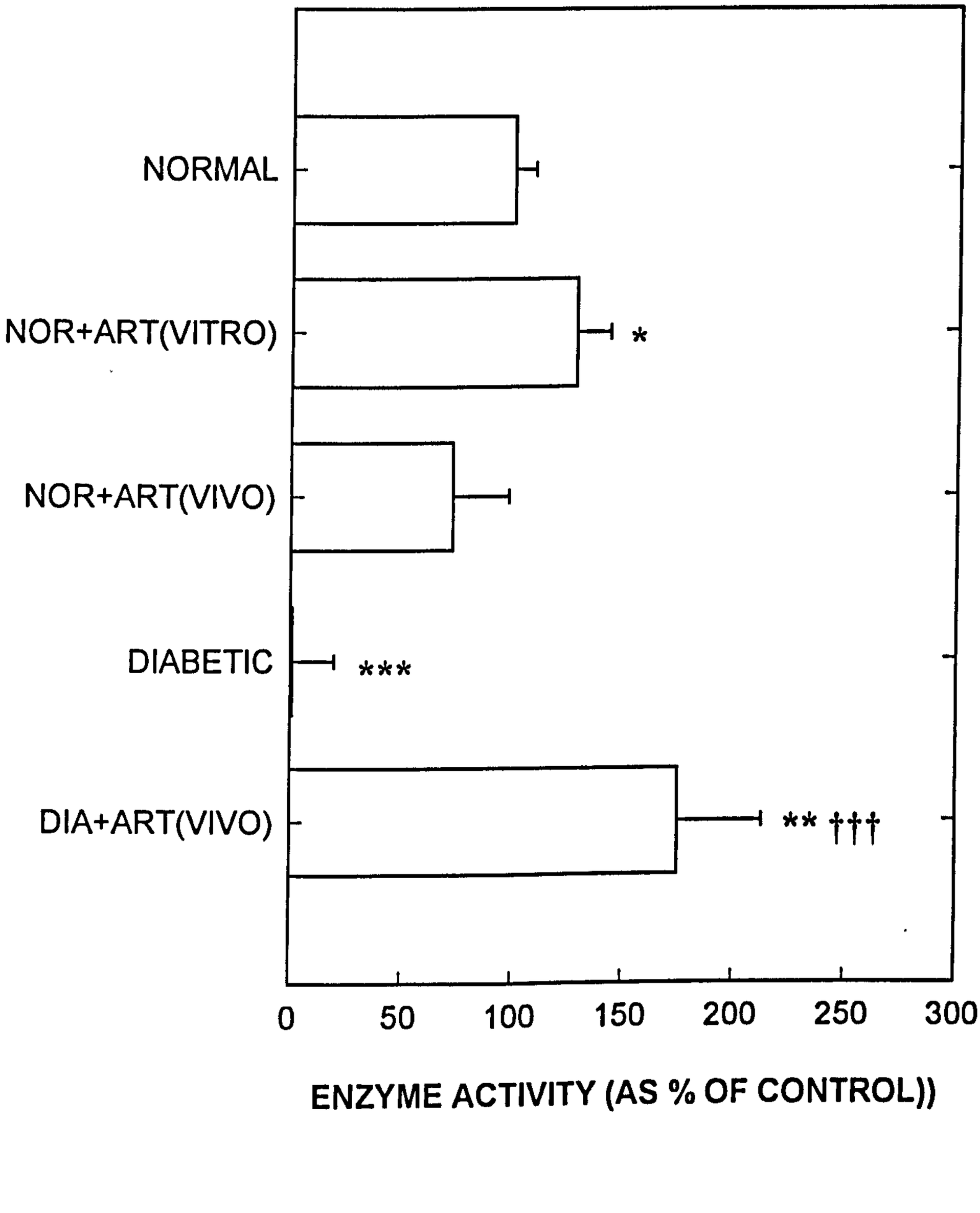

NORMAL
NOR+ART(VITRO)
*
NOR+ART(VIVO)
*
DIABETIC
***
DIA+ART(VIVO)
** †††
0
50
100
150
200
ENZYME ACTIVITY (AS % OF CONTROL)

Figure 4.9 The effect of diabetes and Artemisia extract treatment on 5α-reductase of androst-4-ene3,17-dione by rat liver microsomes.

Control = untreated; NOR + ART.(*VITRO*) = normal rat treated with *Artemisia judaica* in-*vitro*; NOR + ART(*VIVO*) = normal rat treated with *Artemisia judaica* in-*vivo*; DIA + ART(*VIVO*) = diabetic rat treated with *Artemisia judaica* in-*vivo*, * = $p<0.05$, ** = $p< 0.01$ compared to control rat & ††† = $p<0.001$ compared to diabetic rat..

Results expressed as mean ± s.d.(for normal n = 9 and diabetic and diabetic treated with *Artemisia judaica* n = 6)


NORMAL
NOR+ART(VITRO)
*
NOR+ART(VIVO)
DIABETIC
***
DIA+ART(VIVO)
** †††
0
50
100
150
200
250
300
ENZYME ACTIVITY (AS % OF CONTROL))

4.6 Discussion of Results for Liver Parameters

It has previously been shown that induction of the diabetic state causes a decrease in the liver weight (Rouer and Leroy, 1980), and that this may be due to a decreased plasma insulin level resulting in a decrease in hepatic lipid and glycogen storage (Pugazhenthi and Khandelwal, 1990). This would also result in an increase in liver protein content, since if lipid and glycogen storage was decreased and there was therefore a decrease in liver weight, there would be an increase in the amount of protein in each gram of tissue. The results shown here are consistent with previous findings. The change in liver weight was reversed by treatment with *Artemisia* extract.

It has previously been shown that the metabolism of various drugs is strikingly altered by the diabetic state (Defeng *et al.*, 1993). Induction of the diabetic state caused a marked change in the enzyme activity of certain forms of cytochrome P450. These were cytochrome P4502E1, which was increased, and the cytochromes P450 of the 2B and 2C subfamilies, which were decreased. The induction of the diabetic state inhibits the microsomal oxidation of drugs such as aminopyrine by a marked decreased in the activity of the cytochromes P450 of the 2B and 2C subfamilies but increases the oxidation of aniline (Defeng *et al.*, 1993), which is known to be an effective substrate for cytochrome P4502E1. It is possible to measure the enzyme activity of these 3 forms of cytochrome P450 as described by Gibson and Skett (1994). By using the assay of aniline 4-hydroxylase activity described earlier, it is possible to measure the enzyme activity of the 2E1 form of cytochrome P450. The assay of aminopyrine *N*-demethylase used was that described by Nash in 1953 to measure the formation of formaldehyde and is a function of the enzyme activity of the 2B and 2C subfamilies of cytochrome P450.

The overall metabolism of the steroid, androst-4-ene-3,17-dione has been shown to be decreased in diabetic animals (Reinke *et al.*, 1978) although there are reports of increases in some specific enzyme activities (Skett and Joels, 1985). The results presented here are consistent with those found earlier although the increase in 7α-hydroxylase and 5α-reductase seen in earlier work are not seen here. *Artemisia* extract treatment of the diabetic animals reversed or partially reversed the effects of diabetes on aniline, aminopyrine and androst-4-ene-3,17-dione metabolism. The addition of *Artemisia* extract directly to microsomes gave some unexpected results with a

significant increase in aminopyrine *N*-demethylation and androst-4-ene-3,17-dione metabolism indicating that some of the effects of the *Artemisia* extract may be direct on the microsomal enzymes whereas the other effects may be indirect (e.g. that on aniline metabolism). The nature of the active principles of the *Artemisia* extract giving these effects is / are at present unclear.

5. Clinical Experiments

The following results were obtained from a small series of patients examined at the Al-Afia Houn Hospital. Each patient was treated individually as noted and blood glucose was assayed by the technique described in the Materials and Methods.

Patient 1: A man (43 years) with mild diabetes of 5 years duration. This patient had undergone therapy with *Artemisia judaica* and *Marrubium vulgare* with a controlled diet for approximately three years. The patient was asked to stop his own treatment with *Artemisia judaica* and *Marrubium vulgare* extract for the period of the study. His blood glucose levels varied from 162-180 mg/ml before treatment with *Artemisia* extract and 135-159 mg/ml after a 2-7 day period of treatment with *Artemisia* extract (Figure 5.1(1))

Patient 2: A man of 20 years who had been treated with insulin injection (40 i.u. once daily). The patient was then given *Artemisia* extract as described three times daily together with the pre-existing insulin therapy. On insulin alone the blood glucose concentration was 180-192 mg/ml, which fell to 160 mg/ml after 6 days treatment and 107 mg/ml after 9 days treatment with *Artemisia* extract. The insulin dose was subsequently reduced to 20 i.u./day and the blood glucose level rose to 150-152 mg/ml (Figure 5.1 (2)).

Patient 3: A man (age 17 years) who had been treated with plant extracts (one of them being *Artemisia judaica*) since being a juvenile. He had stopped using the *Artemisia* because when he took it he suffered from bradycardia. He had asked the doctor to replace his treatment with lente insulin injection. The patient was given 40 i.u./day insulin. We asked the patient to use *Artemisia* extract with insulin. The patient had polyuria, polydipsia and polyphagia with insulin treatment alone. Blood glucose levels varied between 180 and 210 mg/ml without *Artemisia* extract and declined from 200 to 158 mg/ml over a 5 day treatment period with *Artemisia* extract (Figure 5.1(3)).

Patient 4: A man (aged 24 years) with diabetes of one year duration, He had been treated with diet restriction and insulin (30 i.u. am. and 20 i.u. pm). This was the first time he had used *Artemisia* extract but he knew that the plant is widely used by diabetics. Prior to taking the *Artemisia* extract his blood glucose levels were 231-371 mg/ml and continued to varying widely during a 10 day treatment with the extract (310 mg/ml on day 3 of treatment, 104 mg/ml on day 5, 327 mg/ml on day 8 and 53 mg/ml on day 10) (Figure 5.1(4)).

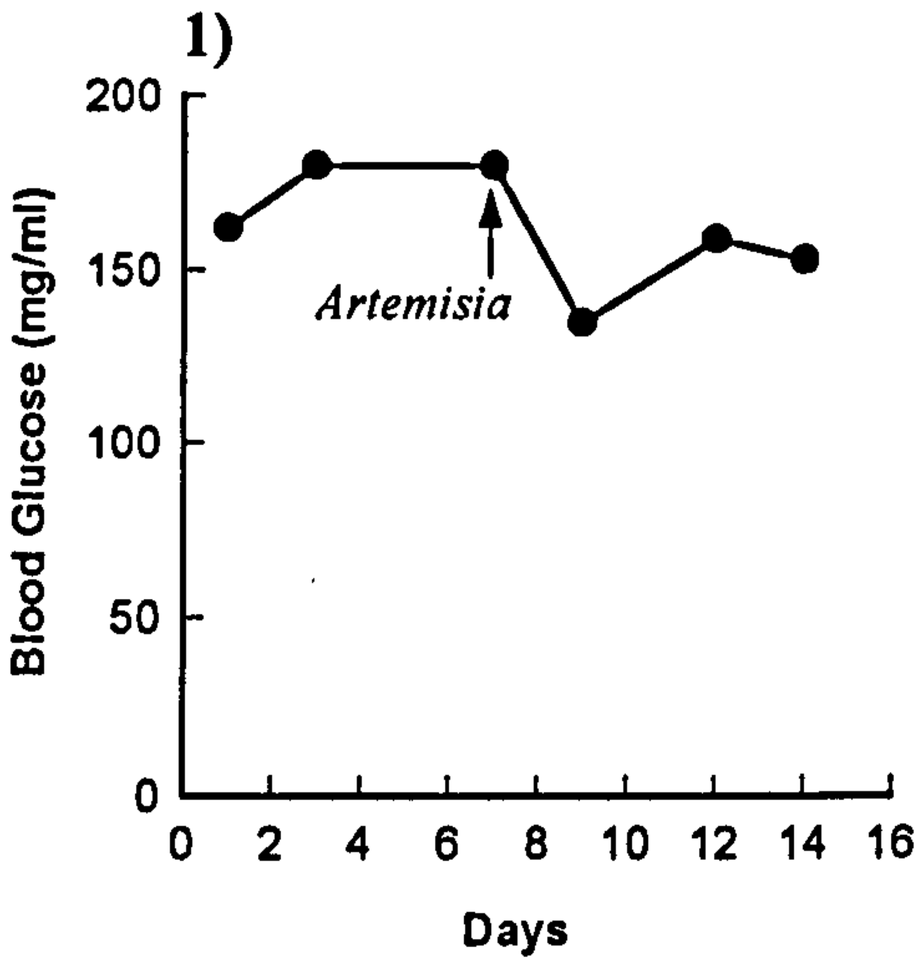
Patient 5: A woman of 55 years with diabetes mellitus of eight years duration. She had used hypoglycaemic agents (Daonil®) and used *Artemisia* and other plants at some times. This patient was asked to stop the self-administration of these plants for the duration of the study. Her blood glucose levels varied from 288-325 mg/ml before administration of *Artemisia* extract and was reduced to 205 mg/ml after 2 days treatment with the extract. Her blood glucose level continued to fall to 180 mg/ml at day 5 and 168 mg/ml on day 7 of treatment (Figure 5.1(5)).

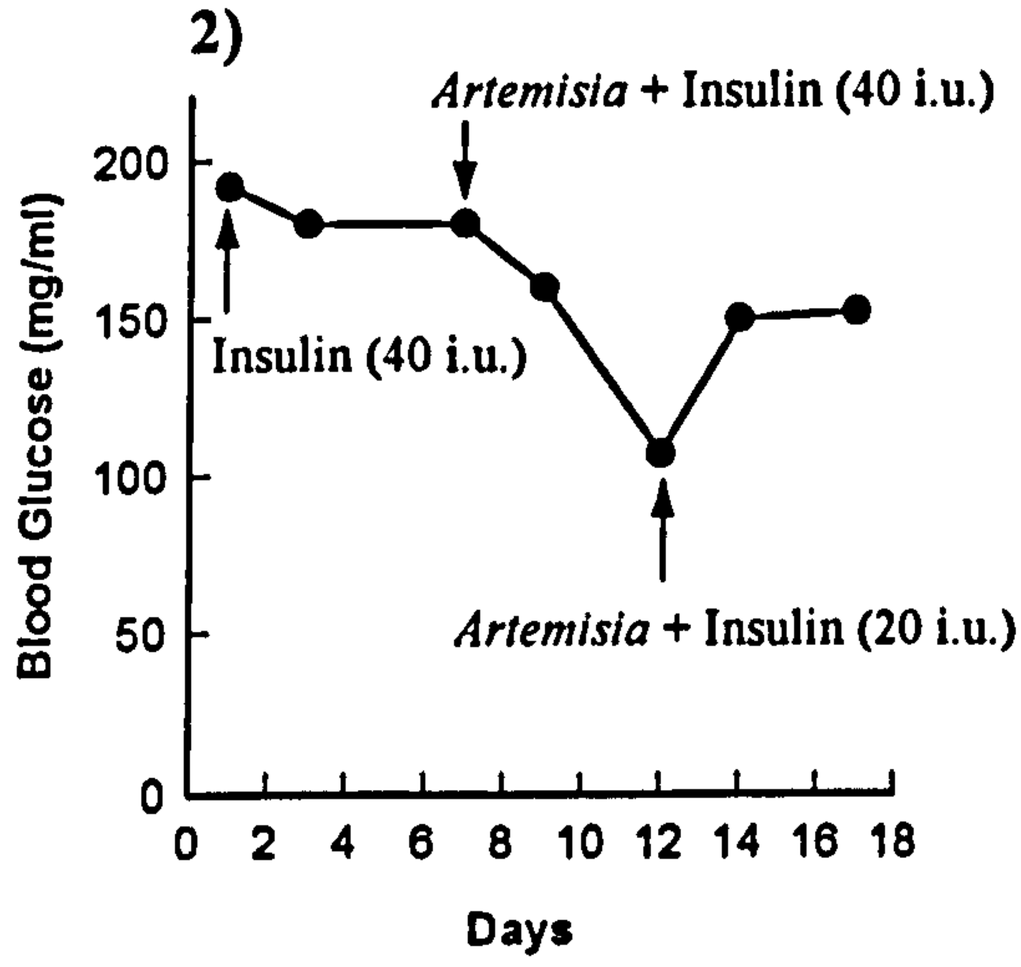
Patient 6: A man of 60 years with mild diabetes of 20 years duration. He had used *Artemisia judaica* extracts for a long time but at irregular intervals and he did not feel any side effects of the treatment. His blood glucose levels were unchanged by treatment with *Artemisia* extract (174-228 mg/ml before and 170-250 mg/ml after) (Figure 5.1(6)).

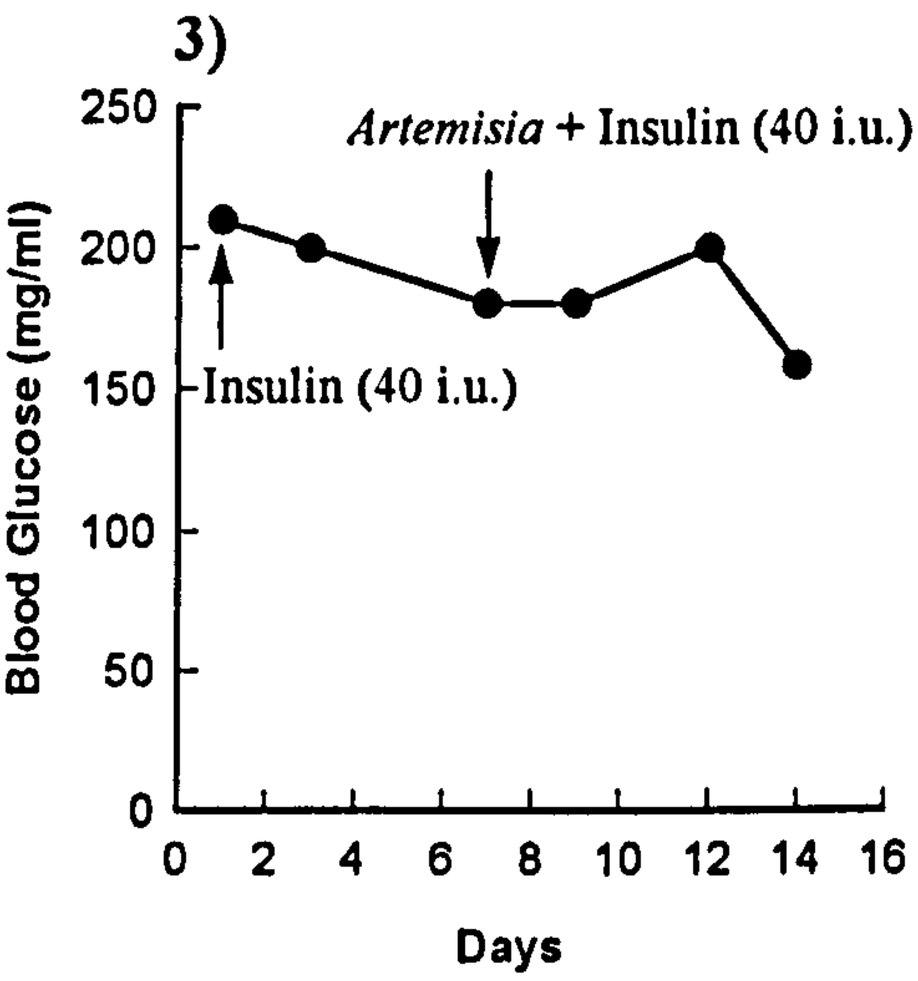
Figure 5.1 Effect of treatment on blood glucose levels.

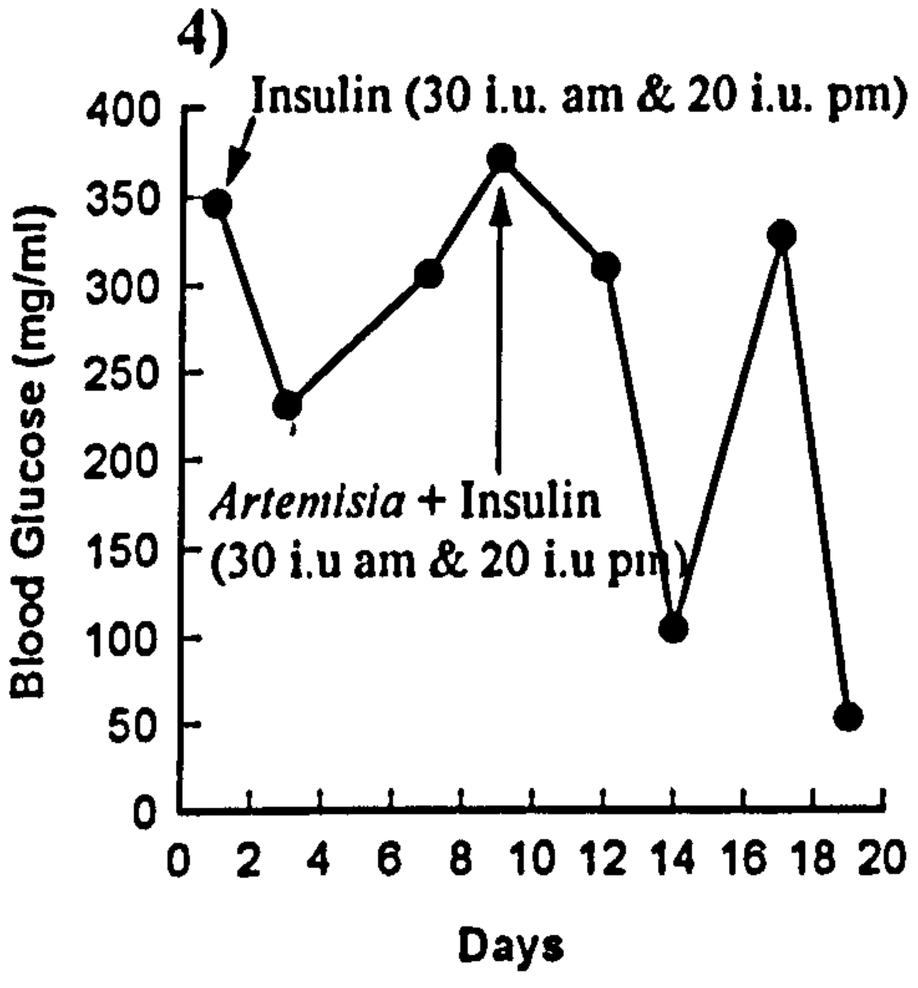
1 = Patient(1), 2 = patient(2), 3 = patient(3),

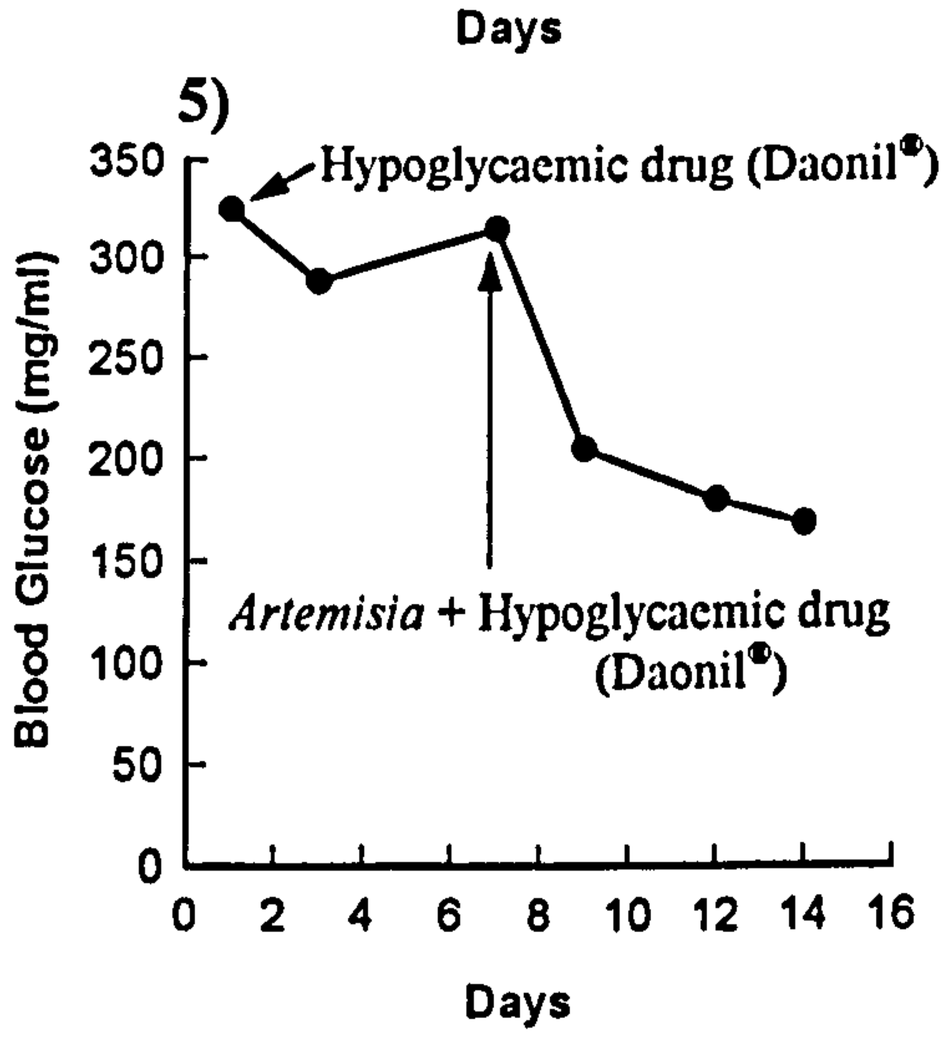
4 = patient(4), 5 = patient(5) & 6 = patient(6)

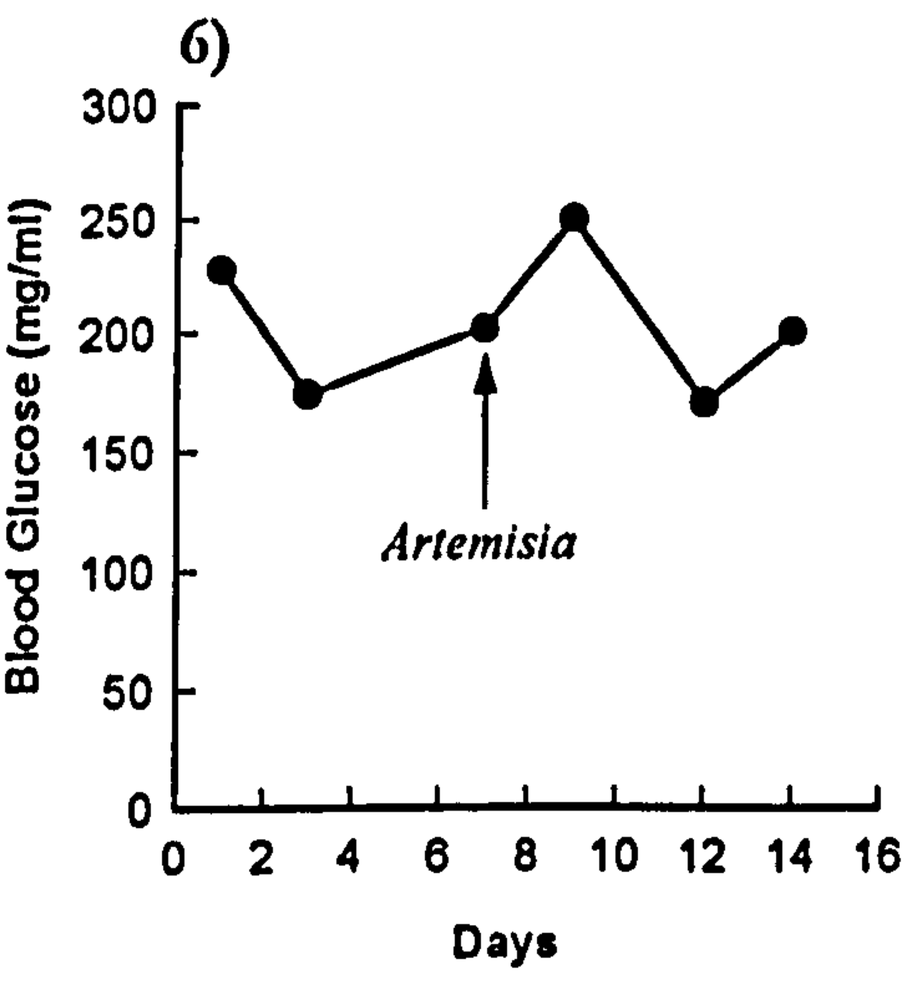

1)
Blood Glucose (mg/ml)
200
150
100
50
0
Artemisia
0 2 4 6 8 10 12 14 16
Days


2)
Artemisia + Insulin (40 i.u.)
Insulin (40 i.u.)
Artemisia + Insulin (20 i.u.)
Blood Glucose (mg/ml)
200
150
100
50
0
0 2 4 6 8 10 12 14 16 18
Days


3)
Artemisia + Insulin (40 i.u.)
Insulin (40 i.u.)
Blood Glucose (mg/ml)
250
200
150
100
50
0
0 2 4 6 8 10 12 14 16
Days


4)
Insulin (30 i.u. am & 20 i.u. pm)
Artemisia + Insulin
(30 i.u am & 20 i.u pm)
Blood Glucose (mg/ml)
400
350
300
250
200
150
100
50
0
0 2 4 6 8 10 12 14 16 18 20
Days


5)
Hypoglycaemic drug (Daonil®)
Artemisia + Hypoglycaemic drug
(Daonil®)
Blood Glucose (mg/ml)
350
300
250
200
150
100
50
0
0 2 4 6 8 10 12 14 16
Days


6)
Artemisia
Blood Glucose (mg/ml)
300
250
200
150
100
50
0
0 2 4 6 8 10 12 14 16
Days

5.1 Discussion:

Several diabetic patients in Libya have reported benefit to their diabetic symptoms of polyuria and polydipsia and a gain in body weight upon daily consumption of the aqueous extract of *Artemisia judaica.* It is well known to most diabetic patients and grows in several countries such as Libya , Egypt, other parts of Africa and the Middle East as well as the United States.

Due to the long history of use of this plant and its efficacy and safety, we were encouraged to perform an evaluation of the effects of *Artemisia judaica* on blood glucose in diabetic patients and also to see whether the extract caused any side effects.

The results showed that *Artemisia judaica* had a potent therapeutic value in treating some patients with diabetes as it significantly reduced blood glucose to near the normal level (80-120 mg/ml). Patients 1, 2 and 4 responded well to *Artemisia* extract but patient 3 had no response after 2 weeks of treatment showing little reduction in blood glucose levels. Patient 4 responded to *Artemisia judaica* except on day 17 where a significant increase to 317 mg/ml in blood glucose concentration was seen. Patient 6 had no response to the treatment with *Artemisia* extract.

The extract seemed to have no side effects except in patient 3 who still suffered from bradycardia but all of the diabetic symptoms had disappeared .

These results suggest that *Artemisia judaica* contains material capable of reducing blood glucose and improving diabetic symptoms but this is only a small-scale clinical trial and much more work, including a full-scale clinical trial, needs to be performed before it can be said that *Artemisia judaica* extract is a worthwhile treatment for diabetes mellitus.

6. *In-Vitro* Experiments (Isolated Hepatocytes)

6.1 Androst-4-ene-3,17-dione Metabolism

6.1.1 The effects of insulin and crude extracts of *Artemisia judaica*

The assay for the metabolism of androst-4-ene-3,17-dione allows the measurement of five different enzyme activities: the 7α-hydroxylase, 6β-hydroxylase, 16α-hydroxylase, 17-oxosteroid oxidoreductase (OHSD) and the 5α-reductase. The data in the tables are expressed as a percentage of relevant control values for each experiment as the control values can vary depending on the animal from which the cells were taken. To avoid this inter-experiment variation, control, and insulin-treated cells were assayed in all experiments to compare to extract-treated cells. The results of the first experiment (1/2 hour & 1 hour) in experiment two are shown in Tables 6.1 and 6.2.

It is seen that neither insulin (at 10^{-6} and 10^{-9}M) nor the crude extract of *Artemisia judaica* had very much effect on steroid metabolism. There were some decreases in 5α-reductase activity with 10^{-9}M insulin ($p<0.001$) and in 7α-, 6β- and 16α-hydroxylase activities with *Artemisia* extract and *Artemisia* extract with insulin (10^{-6}M) ($p<0.05$), but *Artemisia* extract with insulin (10^{-9}M) had no effect on steroid metabolism. This result is in stark contrast to those of Hussin & Skett (1986) who found marked increases in the metabolism of androst-4-ene-3,17-dione in the presence of insulin. The reason(s) for this discrepancy were, thus, investigated.

6.1.2 Effect of animal weight / age

The first difference noticed between the experiments described here and those of Hussin & Skett (1986) was the age (size) of the animals used. Hussin & Skett (1986) used smaller animals (200-250g). The experiment was, thus, repeated using the

Table 6.1: The effect of insulin (10^{-6}M & 10^{-9}M) and *Artemisia judaica* (A10=0.02g/ml, A.j=0.2g/ml) on the 7α-, 6β-, and 16α-, hydroxylase, 17α(β)-hydroxysteroid dehydrogenase and 5α-reductase after <u>1/2 hour</u> of preincubation in hepatocytes obtained from normal rat, p values relative to control values were obtained by ANOVA followed by Dunnet's test * = $p<0.05$, *** = $p<0.001$.
Results are expressed as percentage of the relevant control and mean ± s.d., (n=5).

Enzyme Activity (expressed as % of control)					
GROUP	7α-OH	6βOH	16α-OH	17-OHSD	5α-Red.
Control	100±18.14	100±15.8	100±13.8	100±20.6	100±30.0
Insulin 10^{-6}M	85.9±19.6	96.6±15.7	85.6±15.9	102.4±13.7	86.5±9.80
Insulin 10^{-9}M	72.0±22.5	87.5±43.2	96.3±52.5	94.7±8.60	49.6±8.03***
A10	85.3±10.4	105.0±16.2	105.0±18.2	103.7±19.8	87.6±25.3
A10 + 10^{-6}M	88.2±21.9	103.2±26.3	100±28.4	92.8±25.6	85.7±27.0
A10+ 10^{-9}M	91.6±34.7	95.4±30.1	87.4±25.6	92.5±32.3	95.1±28.5
A.j	61.9±24.4*	70.9±19.9*	65.2±25.3*	99.2±38.7	84.5±42.8
A.j + I^{-6}M	66.31±13.8*	76.3±8.20*	67.95±18.5*	90.3±23.6	75.2±17.1
A.j + I^{-9}M	88.0±14.0	104.7±19.9	95.1±19.9	106.9±31.6	106.0±44.4

Table 6.2: The effect of insulin (10^{-6}M & 10^{-9}M) and *Artemisia judaica* (A.j=0.2, A10=0.02, A100=0.002, A1000=0.0002 g / ml) on the 7α-, 6β- and 16α-, hydroxylase, 17α(β)- hydroxysteroid dehydrogenase and 5α-reductase after <u>1 hour</u> of preincubation in hepatocytes obtained from normal rat, p values relative to control values were obtained by ANOVA followed by Dunnet's test *=p<0.05.
Results are expressed as percentage of the relevant control and mean ± S.D, (n=5).

Enzyme Activity (expressed as % of control)					
GROUP	7α-OH	6βOH	16α-OH	17-OHSD	5α-Red.
Control	100±28.7	100±27.0	100±24.1	100±22.2	100±40.1
Insulin(I^{-6}M)	110.9±29.7	102.6±40.7	121.2±14.7	119.7±24.4	104.3±76.6
Insulin(I^{-9}M)	105.0±32.4	106.2±27.5	85.4±11.9	109.3±24.8	102.4±36.0
A10	107.5±8.50	114.4±12.7	108.8±5.68	116.0±9.26	82.9±15.4
A10 + I^{-6}M	104.8±22.1	95.2±20.6	81.6±13.9	132.0±49.1	94.2±39.8
A10 + I^{-9}M	84.1±26.3	82.1±35.3	76.0±22.4	92.9±45.3	71.9±42.3
A100	132.1±10.7	135.0±4.74	116.7±17.3	130.0±20.3	111.0±40.8
A100 + I^{-6}M	169.7±107.2	77.96±36.0	65.3±25.8	127.6±77.3	126.2±88.4
A100 + I^{-9}M	117.3±26.1	100.9±9.03	97.0±26.1	121.9±39.3	95.9±29.5
A1000	141.5±53.1	135.4±52.4	152.0±53.9	200.8±112.8	100.1±66.7
A1000+ I^{-6}M	100±19.6	110.6±20.5	98.2±21.0	108.6±21.0	88.4±23.6
A1000 + I^{-9}M	99.2±35.9	91.1±32.8	88.5±32.5	97.6±25.2	81.3±37.0

Table 6.3: The effect of insulin (10^{-6}M & 10^{-9}M) and *Artemisia judaica* (A.j=0.2, A10=0.02, A100=0.002, A1000=0.0002 g / ml) on the 7α-, 6β- and 16α-, hydroxylase, 17α(β- hydroxysteroid dehydrogenase and 5α-reductase after 1/2 hour of preincubation in hepatocytes obtained from normal (weighed 200g) rat, p values relative to control values were obtained by ANOVA followed by Dunnet's test $p<0.05$. Results are expressed as percentage of the relevant control and mean ± S.D, (n=5).

Enzyme Activity (expressed as % of control)					
GROUP	7α-OH	6βOH	16α-OH	17-OHSD	5α-Red.
Control	100±14.2	100±6.74	100±9.00	100±11.6	100±14.1
Insulin(I^{-6}M)	142.5±31.3	122.4±44.2	147.7±48.1	155.1±60.2	154.8±76.8
Insulin(I^{-9}M)	111.0±6.11	107.2±1.84	120.2±19.9	105.2±14.2	97.9±15.3
A10	169.4±30.8	119.2±58.8	133.4±75.3	94.1±44.9	112.0±78.8
A10 + I-6M	101.7±25.9	83.1±21.6	84.6±25.6	75.1±12.1	73.5±13.8
A10 + I-9M	119.1±30.1	114.5±23.7	113.2±42.3	75.1±12.8	73.5±13.8
A100	139.9±39.4	110.3±22.6	124.7±20.9	104.9±6.56	100.5±15.0
A100 + I-6M	154.8±30.6*	1423±32.5	146.9±32.0	150.7±30.2	74.7±10.9
A100 + I-9M	133.3±18.8	126.1±27.9	166.5±24.2	131.3±21.3	144.2±15.0
A1000	124.5±14.7	96.1±14.2	115.4±24.0	76.8±13.7	91.3±47.8
A1000+ I-6M	149.1±31.8	126.5±14.1	142.3±30.9	95.4±22.3	89.4±11.6
A1000 + I-9M	125.9±30.1	79.9±17.0	82.7±25.6	97.6±10.1	94.6±16.2

hepatocytes obtained from a 200g male rat as shown in Table 6.3. As is seen, there is still very little effect of insulin or the *Artemisia* extract except that the trend towards an increase in 7α-hydroxylase activity in the presence of *Artemisia* extract has now become significant when 10^{-6}M insulin is added as well. The age of the animal is, thus, of little importance.

6.1.3 Effect of different insulin preparations

The next suggestion was that the insulin, as stored in aliquots in the freezer at -20°C, had degraded. A fresh batch of insulin was made and used directly for addition. As can be seen in Table 6.4, there was still no significant effect of insulin at either concentration. There was very little effect of the *Artemisia* extract except for a significant increase in 6β-hydroxylase activity in the presence of 10^{-9}M insulin. It is unlikely, therefore, that the insulin stability was at fault. In the experiments of Hussin & Skett (1986), a porcine insulin preparation from Novo (Denmark) and Neulente®Zinc insulin (Wellcome, U.K.) was used whereas in these experiments a similar preparation from the Sigma Chemical Co. (U.K.) was used. Could this be the difference? The various insulin preparations and different times were tried in one experiment and the results shown in Tables 6.5a-e. None of the insulin preparations tested had any significant effect on androst-4-ene-3,17-dione metabolism.

6.1.4 Effect of Different Culture Media

The above experiments were performed in Ham's F-10 medium but recent experiments in this laboratory have indicated that this is not the best medium for the maintenance of liver-specific functions in isolated rat hepatocytes (Skett, 1994). It was, thus, decided to test the effects of insulin and *Artemisia* extract in Williams' E medium and in Ham's F-10 in the absence of albumin (in case the insulin was binding to the albumin and being inactivated). As shown in Tables 6.6 and 6.7, neither of these changes altered the lack of effect of insulin or *Artemisia* extract. Although there were some trends to increases and decreases, there was no significant change in the activities measured.

Table 6.4: The effect of insulin (10^{-6}M & 10^{-9}M) and *Artemisia judaica* (A10=0.02 g / ml) on the 7α-, 6β- and 16α-, hydroxylase, 17α(β)- hydroxysteroid dehydrogenase and 5α-reductase after 1/2 hour of preincubation in hepatocytes obtained from normal rat, p values relative to control values were obtained by ANOVA followed by Dunnet's test (* =p<0.05).

Results are expressed as percentage of the relevant control and mean ± S.D, (n=5)

Enzyme Activity (expressed as % of control)					
GROUP	7α-OH	6βOH	16α-OH	17-OHSD	5α-Red.
Control	100±14.1	100±9.71	100±15.7	100±7.28	100±4.94
Insulin(I^{-6}M)	116.9±41.4	90.2±14.6	99.8±14.1	95.6±3.84	93.4±21.4
Insulin(I^{-9}M)	132.6±16.8	117.3±7.81	116.1±7.83	107.7±7.90	90.9±13.7
A10	135.9±31.3	116.7±20.2	110.2±13.1	96.6±3.84	92.4±6.60
A10 + I^{-6}M	115.4±9.70	110.9±4.30	117.2±2.42	98.4±7.82	92.9±6.10
A10 + I^{-9}M	137.9±13.7*	120.7±7.85*	100.1±4.86	101.6±7.60	100.0±20.9

Table 6.5: The effect of different types of insulin (IP=isophane porcine insulin, BInew=bovin insulin new stock & BIold=bovin insulin old stock) on the 7α-, 6β-, and 16α-, hydroxylase, 17α(β)- hydroxysteroid dehydrogenase and 5α-reductase after **a=5min., b=10min., c=15min., d=20min., e=1/2hour** of preincubation in hepatocytes obtained from normal rat, p values relative to control were obtained by ANOVA followed by Dunnet's

. Results are expressed as percentage of the relevant control and mean ± S.D, (n=5)

a)

Enzyme Activity (expressed % of control)					
GROUP	7α-OH	6βOH	16α-OH	17-OHSD	5α-Red.
Control(1/2hr)	100±19.3	100±14.1	100±17.8	100±26.6	100±21.5
IP (10-6M)	89.0±14.8	93.8±12.2	102.3±20.5	113.1±13.3	102.0±22.7
BInew,10^{-6}M	77.1±14.1	78.4±19.5	94.0±24.3	100.5±19.0	98.4±19.9
BIold,10^{-6}M	76.7±22.9	80.7±24.8	92.4±25.3	98.0±16.3	91.6±14.9

b)

Enzyme Activity (expressed as % of control)					
GROUP	7α-OH	6βOH	16α-OH	17-OHSD	5α-Red.
Control(1/2hr)	100±13.96	100±2.24	100±5.64	100±20.1	100±5.99
IP (10-6M)	106.1±11.4	80.9±9.49	75.2±3.39	89.8±11.9	87.6±7.06
BInew,10^{-6}M	118.8±9.94	109.0±4.61	91.1±19.5	99.5±19.0	113.2±15.4
BIold,10^{-6}M	107.7±20.7	101.6±25.7	90.4±22.5	108.6±6.35	110.6±12.1

c)

Enzyme Activity (expressed as % of control					
GROUP	7α-OH	6βOH	16α-OH	17-OHSD	5α-Red.
Control(1/2hr)	100±14.3	100±24.5	100±21.4	100±40.8	100±29.8
IP (10-6M)	95.9±16.4	109.9±9.20	100.6±6.29	102.4±15.9	95.0±17.6
BInew,10^{-6}M	103.6±16.7	100.0±20.3	106.2±22.4	113.7±16.99	96.8±7.97
BIold,10^{-6}M	117.4±27.8	121.2±34.3	124.7±40.4	89.6±25.9	89.5±14.2

d)

Enzyme Activity (expressed as % of control)					
GROUP	7α-OH	6βOH	16α-OH	17-OHSD	5α-Red.
Control(1/2hr)	100±28.6	100±23.8	100±20.7	100±20.3	100±22.5
IP (10-6M)	72.99±6.46	85.5±23.9	79.75±11.9	81.3±17.9	94.8±10.1
BInew,10^{-6}M	82.8±22.75	87.2±22.5	73.1±18.9	81.9±8.43	88.37±17.5
BIold,10^{-6}M	116.3±40.6	87.8±34.6	94.9±31.5	87.0±6.42	81.5±2.95

e)

Enzyme Activity (expressed as % of control)					
GROUP	7α-OH	6βOH	16α-OH	17-OHSD	5α-Red.
Control(1/2hr)	100±19.3	100±14.1	100±17.8	100±26.6	100±21.5
IP (10^{-6}M)	89.0±14.8	93.8±12.2	102.3±20.5	113.1±13.3	102.0±22.7
BInew,10^{-6}M	77.1±14.1	78.4±19.5	94.0±24.3	100.5±19.0	98.4±19.9
BIold,10^{-6}M	76.7±22.9	80.7±24.8	92.4±25.3	98.0±16.3	91.6±14.9

Table 6.6: The effect of insulin (10^{-6}M) and *Artemisia judaica* (A.j=0.2, A10=0.02, A100=0.002, A1000=0.0002, A10000=0.00002 g / ml) on the 7α-, 6β- and 16α-, hydroxylase, 17α(β)- hydroxysteroid dehydrogenase and 5α-reductase after 1/2 hour of preincubation in hepatocytes obtained from normal rat in Ham's F-10 medium, p values relative to control values were obtained by ANOVA followed by Dunnet's test *P = <0.05

Results are expressed as percentage of the relevant control and mean ± S.D, (n=5).

Enzyme Activity (expressed as % of control)					
GROUP	7α-OH	6βOH	16α-OH	17-OHSD	5α-Red.
Control	100±4.40	100±1.89	100±6.43	100±9.81	100±8.76
Insulin($I^{-6}M$)	120±31.3	104.5±20.6	110.8±32.2	83.4±8.60	92.9±9.98
A.j	103.7±18.3	67.2±7.99	83.4±17.7	97.0±20.1	78.0±20.5
A10	104.9±30.5	92.6±0.87	105.3±3.91	98.1±13.9	86.8±2.30
A100	130.0±21.3	82.6±17.0	99.3±20.7	92.0±13.9	99.8±19.7
A1000	135.6±16.4	104.5±44.8	123.3±50.2	75.2±8.75	91.3±4.10
A10000	120.4±11.2*	75.4±5.20*	91.4±1.59	71.7±10.9*	89.2±8.90
A100 + I-6M	119.7±9.20	96.7±6.70	111.7±11.9	104.3±14.1	90.0±7.42
A.j + I-6M	116.0±11.4	68.5±6.70*	74.6±1.40*	92.5±12.2	77.2±3.78*

Table 6.7: The effect of insulin (10^{-6}M & 10^{-9}M) and *Artemisia judaica* (A.j=0.2, A10=0.02 g / ml) on the 7α-, 6β- and 16α-, hydroxylase, 17α(β)- hydroxysteroid dehydrogenase and 5α-reductase after 1/2 hour of preincubation in hepatocytes obtained from normal rat in William's E medium, p values relative to control values were obtained by ANOVA followed by Dunnet's test *P = <0.05.
Results are expressed as percentage of the relevant control and mean ± S.D, (n=5)

Enzyme Activity (expressed as % of control)					
GROUP	7α-OH	6βOH	16α-OH	17-OHSD	5α-Red.
Control	100±27.99	100±30.1	100±27.2	100±12.1	100±7.20
Insulin(I^{-6}M)	79.98±9.50	86.1±2.53	94.8±6.28	101.4±10.8	79.7±3.20
Insulin(I^{-9}M)	92.6±27.9	109.2±41.3	108.7±18.8	121.2±9.68	110.2±1.35
A10	76.1±21.4	73.97±18.02	85.0±4.92	88.5±8.71	78.1±15.0
A.j	86.5±39.2	73.2±9.56*	89.0±3.64	120.8±13.4	103.4±10.6

6.1.5 Effect of different periods of pre-incubation

In the study of Hussin & Skett (1986), it was found that the effect of insulin seen depended on the period during which the cells had been exposed to the hormone. The greatest effect had been seen at ½ hour but had disappeared at 1 hour. The timing of the experiment could, therefore, be crucial. The effect of insulin and *Artemisia* extract was tested at periods ranging from 1/2 to 72 hours. No effect of insulin was seen at any of the time points investigated (Tables 6.8-6.12). The time-dependent effects of *Artemisia* extract were, however, more complex. At ½hr with a 10x diluted extract (0.02 g/ml), there was no effect as noted previously and this lack of effect continued until 4 hours (with a small, though significant, reduction in 6β-hydroxylase activity at 1 hour) (table 6.8). With a more concentrated extract (0.2 g/ml), there was still no effect at 1/2hr but by 1 hour a significant elevation of 16α-hydroxylase and 5α-reductase activity was seen (table 6.9). At 2 hours post-treatment there was a significant increase in 7α-hydroxylase activity. All of these effects had disappeared at 4 hours. A repeat of this experiment with a different cell preparation gave substantially similar results (Table 6.10) with significant increases in 6β- and 16α-hydroxylases and 5α-reductase activities at times after 1 hour. Extension of the period of pre-incubation with *Artemisia* extract up to 72 hours did not result in any enhancement of the action of the extract and insulin (Tables 6.11 and 6.12). There were some significant increases noted (at 24 hours with a x10 diluted extract on 17-OHSD and 5α-reductase activities) but the most significant feature was a marked decrease in enzyme activities at 72 hours post-treatment (down to 50% of control for the 5α-reductase). The length of time during which the cells are in contact with the *Artemisia* extract can, thus, have some bearing on the effects seen but there is still no consistent effect noted.

6.1.6 Effects of Storage of *Artemisia* extract

The crude aqueous extract of *Artemisia judaica* was stored frozen at -20°C in 5ml aliquots. These aliquots were defrosted before addition to the cells. Could the freeze-thawing process have inactivated the extract by destroying the active ingredient? It was decided to test the effects of a fresh and a frozen extract in the same experiment.

Table 6.8: The effect of insulin ($I10^{-6}M$) and *Artemisia judaica* (A10=0.02 g / ml) on the 7α-, 6β- and 16α-, hydroxylase, 17α(β)- hydroxysteroid dehydrogenase and 5α-reductase after 1/2, 1, 2 & 4 hour of preincubation in hepatocytes obtained from normal rat in Ham's F-10 medium, p values relative to control values were obtained by ANOVA followed by Dunnet's test *p <0.05.
Results are expressed as percentage of the relevant control and mean ± S.D (n=5)

Enzyme Activity (as expressed of % of control)					
GROUP	7α-OH	6βOH	16α-OH	17-OHSD	5α-Red.
Control(1/2hr)	100±14.4	100±7.45	100±9.74	100±12.4	100±14.3
A10(1/2hr.)	99.9±11.1	98.6±1.46	93.4±9.80	88.8±9.18	88.7±16.5
Control (1hr)	100±17.7	100±14.9	100±6.28	100±5.26	100±13.3
Insulin($I^{-6}M$) (1hr)	86.9±19.6	102.4±13.7	90.5±9.9	112±20.0	106±12.3
A10 (1hr.)	82.34±18.3	75.8±12.8*	88.4±31.3	91.1±17.6	83.5±22.5
Control (2hr.)	100±20.2	100±19.2	100±23.8	100±4.80	100±22.7
A10 (2hr)	123.1±19.0	119.5±5.70	109.4±10.8	113.6±8.25	97.6±30.7
Control (4hr.)	100±16.9	100±8.82	100±19.5	100±10.3	100±30.9
A10 (4hr.)	93.7±14.96	121.9±18.5*	128.4±24.5	108.6±7.48	90.8±11.8

Table 6.9: The effect of insulin (10^{-6}M) and *Artemisia judaica* (A.j=0.2 g / ml) on the 7α-, 6β- and 16α-, hydroxylase, 17α(β)- hydroxysteroid dehydrogenase and 5α-reductase after 1/2, 1, 2 & 4 hour of preincubation in hepatocytes obtained from normal rat, in Ham's F-10 medium, p values relative to control values were obtained by ANOVA followed Dunnet's test *=p<0.05, ***=p<0.001.

Results are expressed as percentage of the relevant control and mean ± S.D, (n=5)

Enzyme Activity (expressed as % of control)					
GROUP	7α-OH	6βOH	16α-OH	17-OHSD	5α-Red.
Control(1/2hr)	100±17.0	100±11.7	100±2.22	100±8.71	100±5.21
Insulin(I^{-6}M) (1/2hr.)	107.2±2.73	112.0±6.11	105.2±14.2	99.6±12.3	97.9±15.3
A.j(1/2hr.)	82.4±10.3	99.2±9.10	102.2±9.20	104.2±8.70	102.4±5.50
Control (1hr)	100±2.50	100±12.2	100±22.7	100±4.70	100±16.2
A.j (1hr.)	102.1±11.4	114.11±3.40	132.5±10.9*	108.7±8.80	128.9±15.8*
Control (2hr.)	100±8.10	100±7.80	100±10.5	100±14.8	100±17.3
Insulin(I^{-6}M)	105.5±32.4	109.2±24.8	103.2±26.5	102.5±36.0	85.4±11.9
A.j (2hr) (2hr.)	137±13.3***	119.5±33.3	139.6±33.0*	110.8±10.4	103.7±25.0
Control (4hr.)	100±18.0	100±7.30	100±26.4	100±3.13	100±19.2
A.j (4hr.)	115.0±13.6	107.8±23.3	76.7±23.3	99.5±7.00	133.7±39.6

Table 6.10: The effect of Insulin (10^{-6}M) and *Artemisia judaica* (A.j=0.2 g / ml) on the 7α-, 6β-, and 16α-, hydroxylase, 17α(β)- hydroxysteroid dehydrogenase and 5α-reductase after 1/2, 1, 2 & 4 hour of preincubation in hepatocytes obtained from normal rat in William 'E medium, p values relative to control values were obtained by ANOVA followed by Dunnet's' test, *=p0.05, ***=p<0.001.
Results are expressed as percentage of the relevant control and mean ± S.D, (n=5).

Enzyme Activity (expressed as % of control)					
GROUP	7α-OH	6βOH	16α-OH	17-OHSD	5α-Red.
Control(1/2hr)	100±16.8	100±12.1	100±7.70	100±7.81	100±12.6
A.j(1/2hr.)	122.7±25.2	111.1±10.4	110.7±9.80	100±6.0	123.0±17.8
Control (1hr)	100±9.52	100±12.1	100±18.8	100±16.4	100±23.4
Insulin (1hr)	102.7±23.0	110.3±11.4	100.7±13.80	89±7.0	113.0±7.8
A.j (1hr.)	109.1±18.9	140.8±7.5***	129.7±12.8*	113.1±18.5	126.8±18.7*
Control (2hr.)	100±21.6	100±3.36	100±9.34	100±11.28	100±11.0
Insulin (2hr)	110.1±9.04	85.8±9.47	103.9±7.3	92.7±13.0	104.0±20.5
A.j (2hr)	112.1±6.04	95.8±3.47	143.9±4.3***	112.7±23.5	104.0±17.5
Control (4hr.)	100±8.17	100±21.1	100±3.0	100±21.0	100±18.0
Insulin (4hr)	10.2±16.8	85.9±14.7	112.9±9.30	121.7±19.5	100.4±6.7
A.j (4hr.)	112.0±46.96	85.49±24.7	120.9±4.26	112.7±23.5	133.1±2.7**

Table 6.11: The effect of Insulin (10^{-6}M) and *Artemisia judaica* (A10=0.02 g / ml) on the 7α-, 6β-, and 16α-, hydroxylase, 17α(β)- hydroxysteroid dehydrogenase and 5α-reductase after 24, 48 & 72 hour of preincubation in hepatocytes obtained from normal rat, p values relative to control values were obtained by ANOVA followed by Dunnet's test **=p<0.01 and p<0.001.
Results are expressed as percentage of the relevant control and mean ± S.D, (n=5).

Enzyme Activity (expressed as % of control)					
GROUP	7α-OH	6βOH	16α-OH	17-OHSD	5α-Red.
Control(24hr)	100±20.1	100±18.1	100±28.6	100±12.6	100±8.16
Insulin (24hr)	116.4±5.6	100.7±15.2	101.0±11.5	109.8±17.6	99.0±10.1
A10 (24hr.)	106.0±6.57	116.7±11.3	111.±10.9	133.8±10.9**	163.0±19.0**
Control (48hr)	100±37.8	100±12.2	100±6.44	100±13.3	100±12.1
A10 (48hr.)	80.69±23.8	127.3±4.20**	110.8±23.1	103.9±5.70	126.0±33.7
Control (72hr)	100±30.1	100±9.90	100±22.7	100±9.15	100±20.8
A10 (72hr)	66.1±28.9	55.9±7.18***	83.2±10.5	64.0±5.50***	51.7±11.9**

Table 6.12: The effect of Insulin (10^{-6}M) and *Artemisia judaica* (A.j=0.2 g / ml) on the 7α-, 6β- and 16α-, hydroxylase, 17α(β)- hydroxsteroid dehydrogenase and 5α-reductase after 24, 48 & 72 hour of preincubation in hepatocytes obtained from normal rat, p values relative to control values were obtained by ANOVA followed by Dunnet's. Results are expressed as percentage of the relevant control and mean ± S.D, (n=5).

Enzyme Activity (expressed as % of control)					
GROUP	7α-OH	6βOH	16α-OH	17-OHSD	5α-Red.
Control(24hr)	100±12.1	100±10.5	100±11.8	100±4.30	100±16.5
Insulin (24hr)	106.1±5.60	90.5±15.8	99.5.±7.20	96.4±8.58	98.5±11.3
A.j (24hr.)	86.0±2.50	92.5±7.80	87.4.±8.20	88.3±2.85	88.9±10.6
Control (48hr)	100±11.2	100±9.40	100±15.1	100±13.2	100±10.7
A.j (48hr.)	85.7±5.40	86.5±6.30	105.5±12.3	94.7±5.60	98.8±7.20
Control (72hr)	100±57.1	100±16.1	100±14.7	100±1.80	100±5.30
A.j (72hr)	86.3±8.70	91.4±2.90	101.3±18.8	119.9±23.1	90.7±7.40

The results are shown in Table 6.13. There are no significant changes in any of the enzyme activities tested with either the fresh or the frozen extract. The result of this experiment is, thus, inconclusive.

The overall outcome of this series of experiments is disappointing. It was impossible to reproduce the results of Hussin & Skett (1986) with respect to the effects of insulin on androst-4-ene-3,17-dione metabolism. The reasons for this are unclear and require further examination. This means that the results obtained with the crude aqueous extract of *Artemisia judaica* are difficult to interpret. We cannot compare the effects of the extract to that of insulin if the insulin itself is not giving the expected effect.

6.1.7 The effect of Preincubation with *Marrubium vulgare* extract :

Exposure of the hepatocytes to different concentrations of a crude aqueous extract of *Marrubium vulgare* gave little change in the metabolism of androst-4-ene-3,17-dione except at high concentration (0.2 g/ml) where a significant decrease in enzyme activities of the 7α-, 6β- and 16α-hydroxylases was seen at 1 hour and smaller effect at 1/2 hour. (Table 6.14).

6.1.8 The effect of Pre-incubation with *Anvillea carcinii* :

Table 6.15 shows the effect of a crude extract of *Anvillea carcinii* on the metabolism of androst-4-ene-3,17-dione in isolated rat hepatocytes. It is seen that there is no change when different concentrations of *Anvillea* extract are used except that after 1 hour *Anvillea* extract at a concentration of 0.02 g/ml caused a significant decrease in all enzyme activities. By 4 hours post-treatment, the effects had either disappeared or reversed with significant increases in 17-OHSD and 5α-reductase activities (Table 6.15).

Table 6.13: The effect of *Artemisia judaica* (A10=0.02, A.j=0.2 g / ml) on the 7α-, 6β-, and 16α-, hydroxylase, 17α(β)- hydroxysteroid dehydrogenase and 5α-reductase after 1/2 hour of preincubation in hepatocytes obtained from normal rat, p values relative to control values were obtained by ANOVA followed by Dunnet's test Results are expressed as percentage of the relevant control and mean ± S.D, (n=5)

Enzyme Activity (expressed as % of control)					
GROUP	7α-OH	6βOH	16α-OH	17-OHSD	5α-Red.
Control	100±23.5	100±29.3	100±34.8	100±24.6	100±37.8
A10 (fresh)	81.4±15.3	77.6±15.0	80.3±12.7	95.2±11.1	77.4±12.7
A10 (frozen)	82.4±19.9	90.8±22.7	92.7±14.5	93.4±21.4	79.8±23.8
A.j (fresh)	86.5±17.8	119.3±32.8	95.9±25.7	105.9±17.5	89.4±24.0
A.j (frozen)	100±26.7	92.7±18.1	96.8±13.8	115.3±23.8	79.7±17.20

Table 6.14: The effect of *Marrubium vulgare* (M=0.2, M10=0.02, M100=0.002, M1000=0.0002, M10000=0.00002 g / ml) on the 7α-, 6β-, and 16α-, hydroxylase, 17α(β)- hydroxysteroid dehydrogenase and 5α-reductase after 1/2 hour of preincubation in hepatocytes obtained from normal rat, p values relative to control values were obtained by ANOVA followed by Dunnet's * =P<0.05 and *** =p<0.001. Results are expressed as percentage of the relevant control and mean ± S.D, (n=5).

Enzyme Activity (expressed as % of control)					
GROUP	7α-OH	6βOH	16α-OH	17-OHSD	5α-Red.
Control(1/2hr)	100±14.1	100±14.6	100±12.7	100±11.8	100±11.8
M(1/2hr.)	91.6±11.8	63.3±8.60**	68.7±8.40**	101.3±17.3	120.1±24.9
M10 (1/2hr.)	100.9±25.3	100.4±13.3	110.4±10.9	103.6±13.5	102.8±10.0
M100(1/2hr)	107.6±14.71	102.9±8.92	110.3±11.3	110.6±21.2	105.9±12.8
M1000(1/2hr)	99.3±18.6	99.4±12.5	100.97±11.58	110.6±24.6	90.8±1.95
M10000(1/2hr	127.9±14.8	114.8±4.00	129.9±6.90	128.8±28.2	118.5±23.1
Control (1hr.)	100±42.1	100±39.8	100±33.9	100±11.24	100±20.5
M (1hr.)	40.97±8.85**	26.69±7.35**	22.17±2.43**	65.7±4.73	120.1±36.3
M10 (1hr.)	76.6±13.6	54.58±5.50	54.4±9.80	86.8±16.5	110.5±25.5
Control(4hr.)	100±18.0	100±21.2	100±18.6	100±13.3	100±24.7
M (4hr.)	115.4±23.97	87.6±22.1	113.4±18.2	121±29.67	108.9±5.98
M10 (4hr.)	101.3±19.8	96.6±13.8	88.2±8.25	115.6±7.90	96.3±23.9

Table 6.15: The effect of *Anvillea carcinii* (V=0.2, V10=0.02, V100=0.002, V1000=0.0002, V10000=0.00002 g / ml) on the 7α-, 6β-, and 16α-, hydroxylase, 17α(β)- hydroxysteroid dehydrogenase and 5α-reductase after 1/2 hour of preincubation in hepatocytes obtained from normal rat, p values relative to control values were obtained by ANOVA followed by Dunnet's test, ** =p<0.01 and *** =p<0.001. Results are expressed as percentage of the relevant Control and Mean ± S.D, (n=5)

Enzyme Activity (expressed as % of control)					
GROUP	7α-OH	6βOH	16α-OH	17-OHSD	5α-Red.
Control(1/2hr)	100±14.1	100±14.6	100±12.7	100±11.8	100±11.8
V(1/2hr.)	109.8±85.4	85.4±6.30	97.8±7.85	107.6±17.1	108.4±17.7
V10 (1/2hr.)	108.9±14.2	93.3±6.61	112.4±9.01	114.0±38.3	107.9±12.6
V100(1/2hr)	106.2±15.5	115.8±17.5	111.3±15.8	113.4±23.7	105.9±12.8
V1000(1/2hr)	104.0±15.5	89.2±16.2	92.7±13.4	120.9±3.30	97.7±17.0
V10000(1/2hr	111.1±11.3	97.9±9.22	101.7±6.50	112.9±23.4	105.9±5.13
Control (1hr.)	100±42.1	100±39.8	100±33.9	100±11.24	100±20.5
V (1hr.)	86.3±20.7	88.1±23.8	87.7±20.6	101.9±29.6	85.3±18.2
V10 (1hr.)	39.4±12.4***	30.0±7.81***	32.2±5.09***	53.2±11.4**	38.1±6.20***
Control(4hr.)	100±18.0	100±21.2	100±18.6	100±13.3	100±24.7
V (4hr.)	95.7±0.66	93.1±17.2	123.1±24.4	193.7±33.4**	178.8±32.7*
V10 (4hr.)	110±26.9	67.4±15.7	85.6±41.2	111.5±63.9	123.9±57.3

6.2 Discussion of steroid metabolism

In 1986, Skett found that STZ-induced diabetes mellitus could affect steroid metabolism in the rat liver and that this effect could be reversed by insulin administration to the diabetic animals. Steroid metabolism is closely related to drug metabolism in the rat liver by the enzymes involved and by an apparent common control mechanism, thought to be mediated via the pituitary gland (Skett *et al.*, 1984). As it was not certain how diabetes affected liver function in this case (e.g. directly via changes in blood insulin levels or indirectly via the pituitary gland as above), it was decided to study the effect of insulin on androst-4-ene-3,17-dione metabolism in primary culture of rat hepatocytes. As the biological effects of insulin vary in their time of onset from very rapid responses (seconds to minutes) to quite slow modulations of cell proliferation, that may take hours to be expressed the experiments were conducted over a time period ranging from 1/2 hour to 72 hours.

Androst-4-ene-3,17-dione was used as a substrate in these experiments because it has a sex-dependent metabolism which is well characterized (Stenberg, 1976) and also because it has commonly been used in this laboratory and its metabolites are well-defined and are easily separated and its labelled and unlabelled forms are easily available. Only male rats were used in all of the experiments because it was found that the effect of diabetes mellitus on steroid metabolism was sex-dependent i.e. only seen in the male. It was thought that diabetes interfered with the androgenic stimulation of drug metabolism in the male (Dixon *et al.*, 1961; Skett, 1986) and, therefore, drug metabolism would be unaffected by the same treatment in the female (Skett, 1986). It was decided to use the same hepatocytes approach in our studies because of the above. It was also thought appropriate as the hepatocytes can be obtained in high yields and one can do more ,and long term hormonal, studies. This is in contrast to the perfused liver where only one or two preparations may be run at one time, the technique requires a high degree of skill and the preparations can only be kept viable for a few hours.

We used Ham's F-10 supplemented with 0.1% bovine serum albumin as a nutrient medium because it was found that the enzyme activities were well maintained and that steroid metabolising enzyme activities returned to control level after 2 days in

culture (Hussin & Skett, 1986). We used insulin 10^{-6}M in most of the experiments because Hussein was found the maximum effect was reached at this concentration.

Insulin has, been demonstrated to affect the metabolism of androst-4-ene-3,17-dione *in-vivo* (Skett, 1986) and, in 1988, Hussin & Skett showed that insulin had a direct effect on steroid metabolism in rat hepatocytes by increasing the activity of all enzymes, unlike *in-vivo* where selective increases in enzyme activities were seen. Insulin (10^{-6}M) was used in most of the experiments as Hussin & Skett (1988) had found the maximum effect was reached at this concentration.

The initial purpose of the present study was to test if extracts of the plants, *Artemisia judaica, Marrubium vulgare* and *Anvillea carcinii*, which are used as antidiabetics in Libya, could have the same effect as insulin on hepatocytes isolated from normal rats.

Initially, after 1/2 an hour exposure to insulin at concentrations of 10^{-6} and 10^{-9}M, *Artemisia* extract and *Artemisia* extract with insulin, there was no significant increase in any of the enzyme activities studied and, indeed, a significant decrease was found with *Artemisia* extract with insulin. This data is not in agreement with the results of Hussin & Skett (1988), who found that there was a significant increase in all of the enzyme activities at all concentrations of insulin.

Attempts to ascertain the reason(s) for this discrepancy in the results by a close examination of the methods of Hussin & Skett (1986: 1988) and comparison with the techniques used in this study have proved inconclusive. Changing the pre-incubation period, testing the preparations of insulin and extract used, changing the incubation medium and altering the weight/age of the animals made very little difference. The basis of the discrepancy between the results of this study and those of Hussin & Skett remains unresolved.

When we did not find any positive results with *Artemisia* extract, we used other plant extracts, from *Marrubium vulgare* and *Anvillea carcinii* in different concentration and different times. Neither of these extracts caused any significant increases in enzyme activity although, rarely, significant decreases were seen at some concentrations of extract.

The assay of hepatic metabolism of androst-4-ene-3,17-dione does not, therefore, seem to be of use in detecting insulin-like activity in the system employed in this study.

6.3 *Glycogen Phosphorylase* a

6.3.1 Effect of crude extracts of medicinal plants on the activity of glycogen phosphorylase *a* in hepatocytes isolated from normal rat hepatocytes:

The graphs (Figure 6.1 and 6.2) and tables (Tables 6.17-6.26) show the effects of tested compounds (insulin, glucagon and crude extracts of plants (*Artemisia judaica, Anvillea carcinii & Marrubium vulgare*) on glycogen phosphorylase *a* activity in isolated rat hepatocytes. In these experiments insulin and glucagon were used as standards to compare to the activity of the crude plant extracts to see any insulin-like or glucagon-inhibiting activity. Results are expressed as percentage change from control. Figure 6.1a shows that insulin had little effect on glycogen phosphorylase *a* activity in hepatocytes isolated from control animals at all different concentrations except at 10^{-5}M, which significantly decreased the activity ($p<0.001$). As shown in Figures 6.1b-d , the presence of insulin (10^{-6}M), which itself did not decrease enzyme activity, with different concentrations of crude plant extracts significantly decreased the glycogen phosphorylase *a* activity in virtually all cases. The plant extracts alone were less able to decrease glycogen phosphorylase *a* activity although significant decreases were seen with *Artemisia* extract (2×10^{-1} and 2×10^{-4}g/ml), *Anvillea* extract (2×10^{-6} 10^{-4}g/ml) and *Marrubium* extract (2×10^{-4}g/ml). The crude plant extracts alone were never as potent as the insulin and plant extract mixture except for the highest dose of *Artemisia*

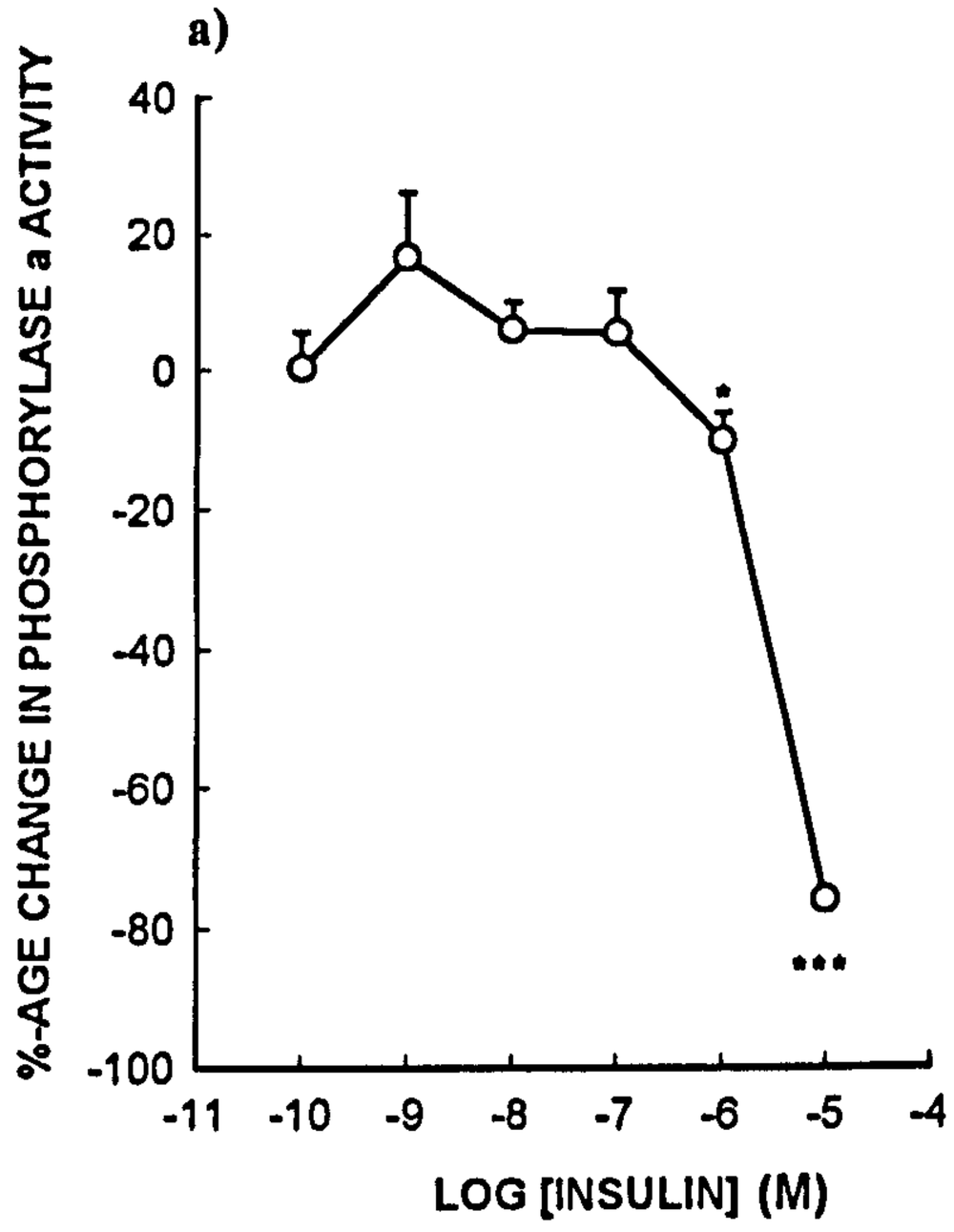
extract. Under similar experimental conditions the presence of glucagon (10^{-6} - 10^{-10}M) had a biphasic effect on glycogen phosphorylase *a* activity (Figure 6.2a). At 10^{-10} - 10^{-9}M, glucagon decreased glycogen phosphorylase *a* activity whereas at 10^{-8} - 10^{-7}M a marked increase was seen. At 10^{-6}M glucagon, a return to a significant decrease was seen. If crude plant extracts alone were tested (Figures 6.2b-d), it is seen

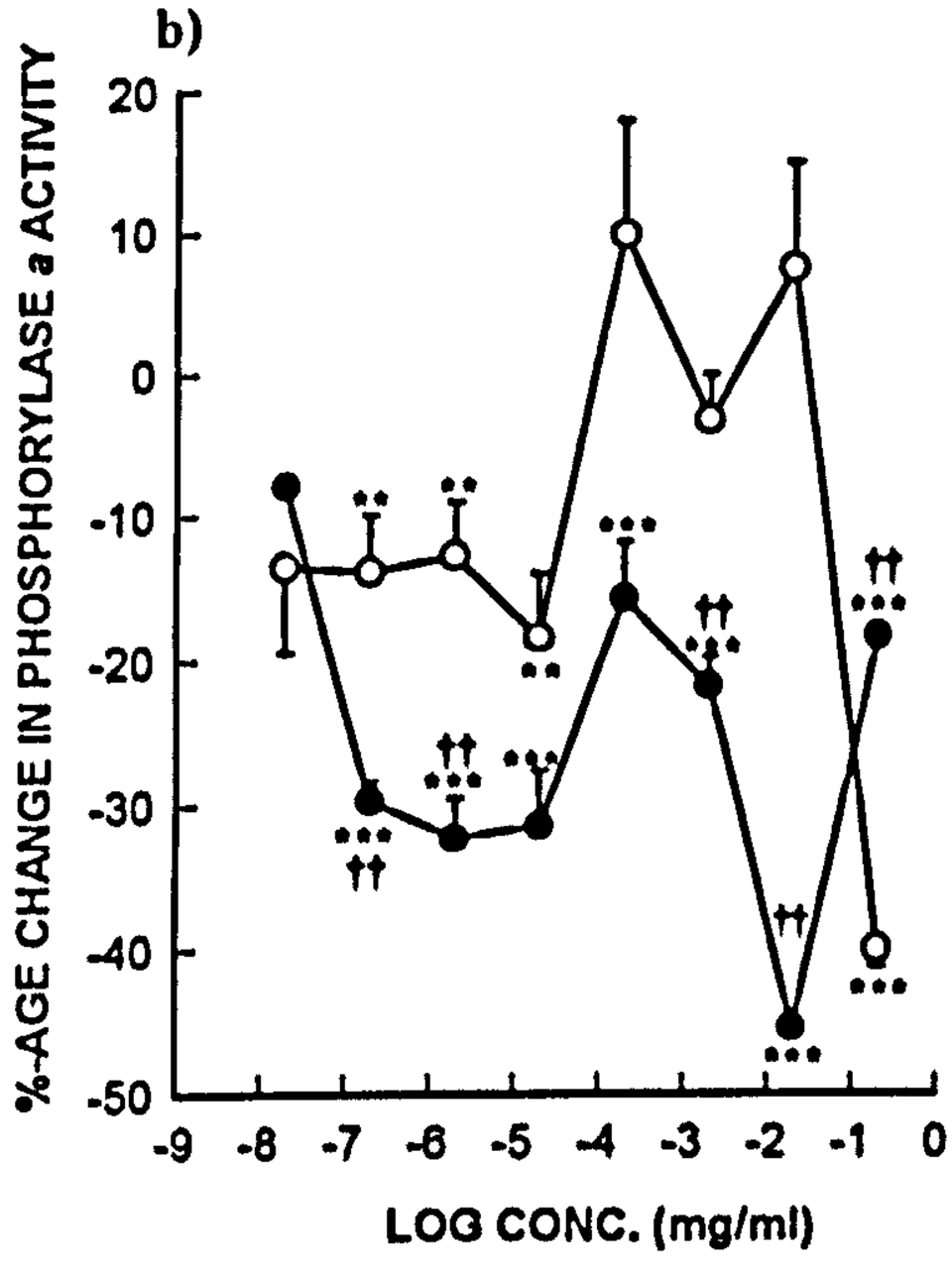
Figure 6.1: Dose-response curves for the effectinsulin and extracts of *Artemisia judaica* (b); *Marrubium vulgare* (c) and *Anvillea carcinii* (d) on glycogen phosphorylase *a* (a) in isolated rat hepatocytes preincubated for 30 min in Krebs-Henseleit medium and the effect of 3 min exposure to the extracts and hormone on the enzyme activity measured, compared to basal phosphorylase activity * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, and † = $p<0.05$, †† = $p<0.01$, ††† = $p<0.001$ compared to respective dose of extract alone, p were obtained by ANOVA followed by Dunnet's test.

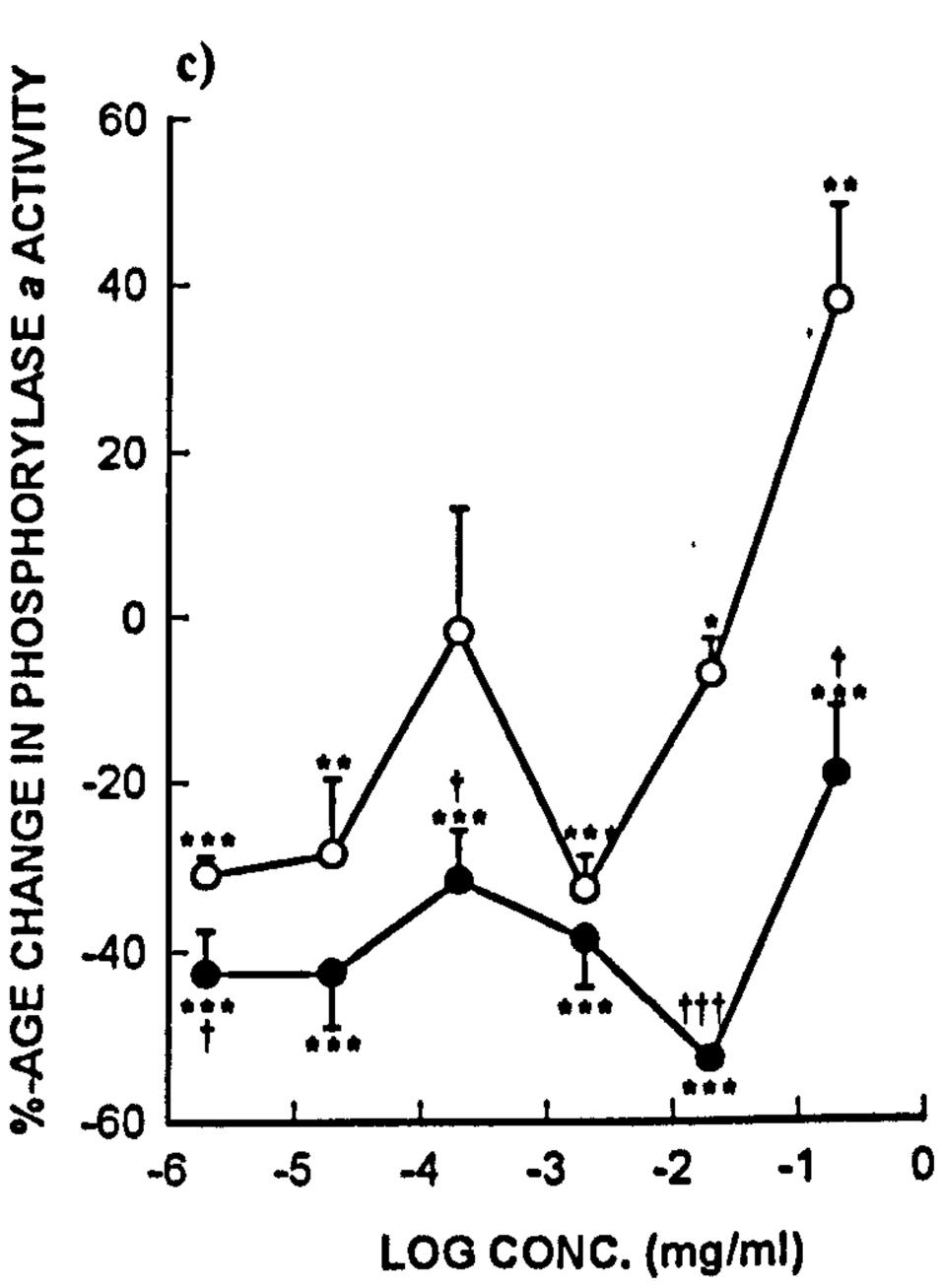
Results are expressed as mean ± s.d. (n=3).

o = Insulin, *Artemisia*, *Marrubium* & *Anvillea* alone

• = *Artemisia*, *Marrubium* or *Anvillea* + insulin (10^{-6}M)


a)
%-AGE CHANGE IN PHOSPHORYLASE a ACTIVITY
40
20
0
-20
-40
-60
-80
-100
-11
-10
-9
-8
-7
-6
-5
-4
LOG [INSULIN] (M)


b)
%-AGE CHANGE IN PHOSPHORYLASE a ACTIVITY
20
10
0
-10
-20
-30
-40
-50
-9
-8
-7
-6
-5
-4
-3
-2
-1
0
LOG CONC. (mg/ml)


c)
%-AGE CHANGE IN PHOSPHORYLASE a ACTIVITY
60
40
20
0
-20
-40
-60
-6
-5
-4
-3
-2
-1
0
LOG CONC. (mg/ml)

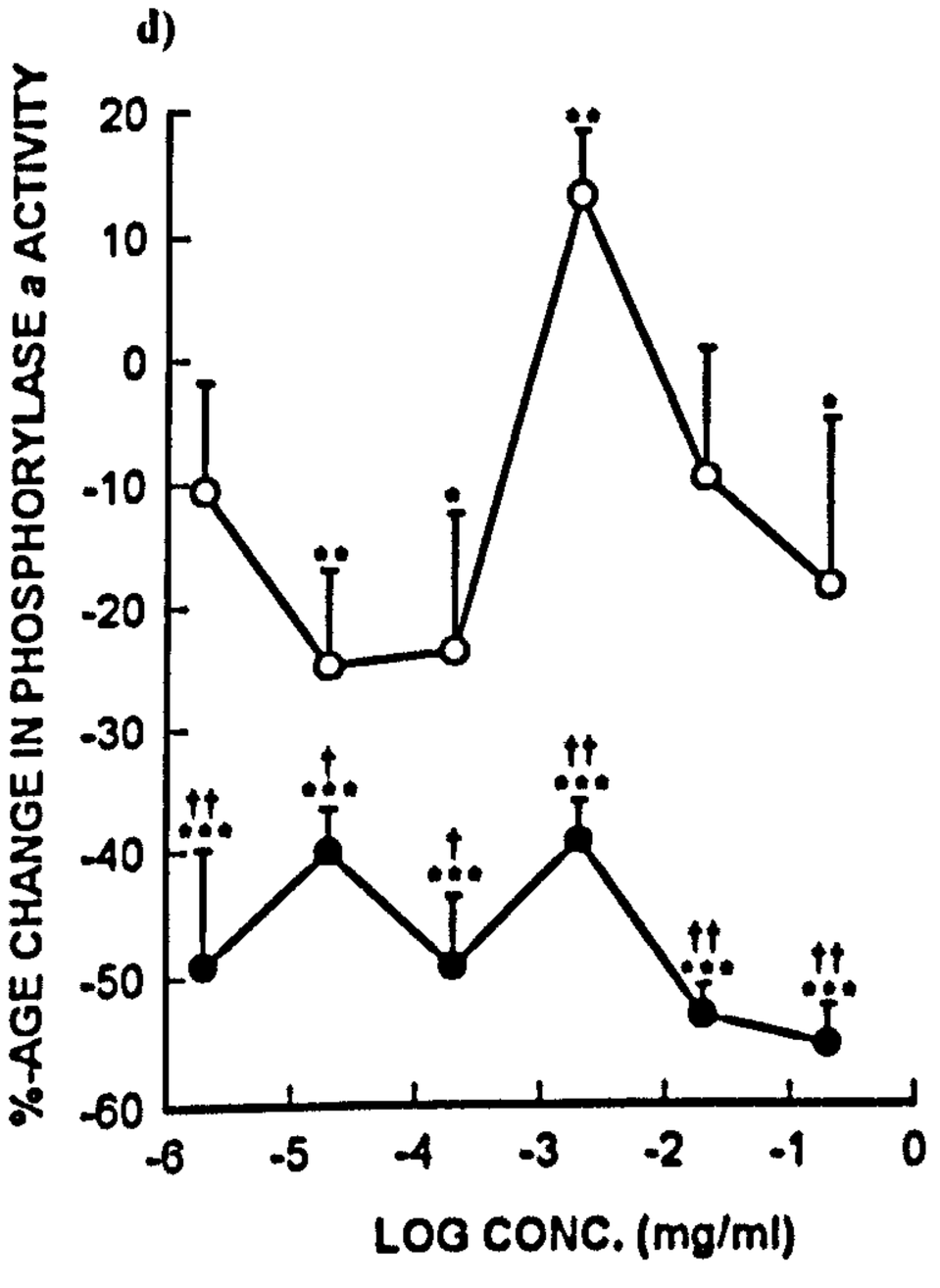
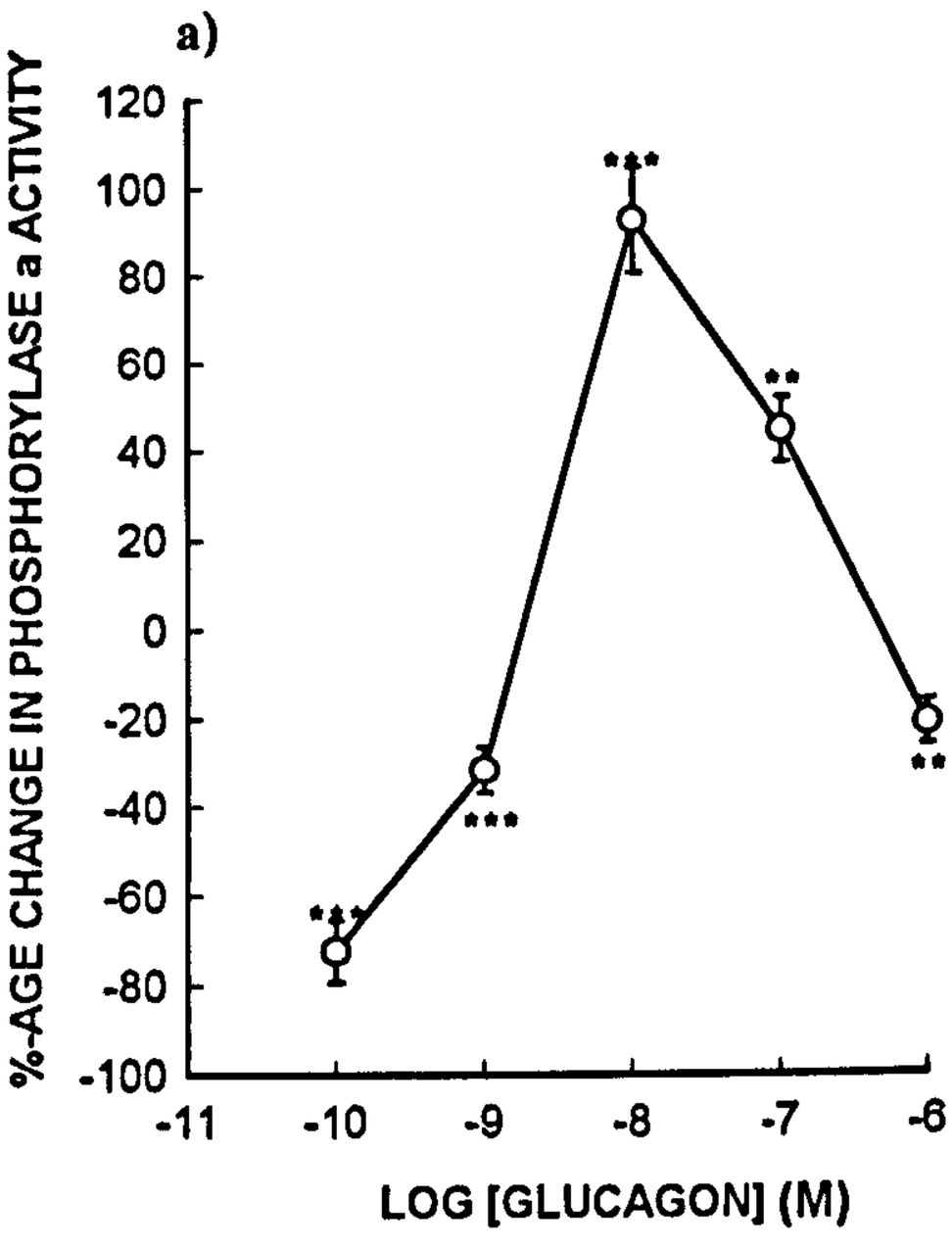

d)
%-AGE CHANGE IN PHOSPHORYLASE a ACTIVITY
20
10
0
-10
-20
-30
-40
-50
-60
-6
-5
-4
-3
-2
-1
0
LOG CONC. (mg/ml)

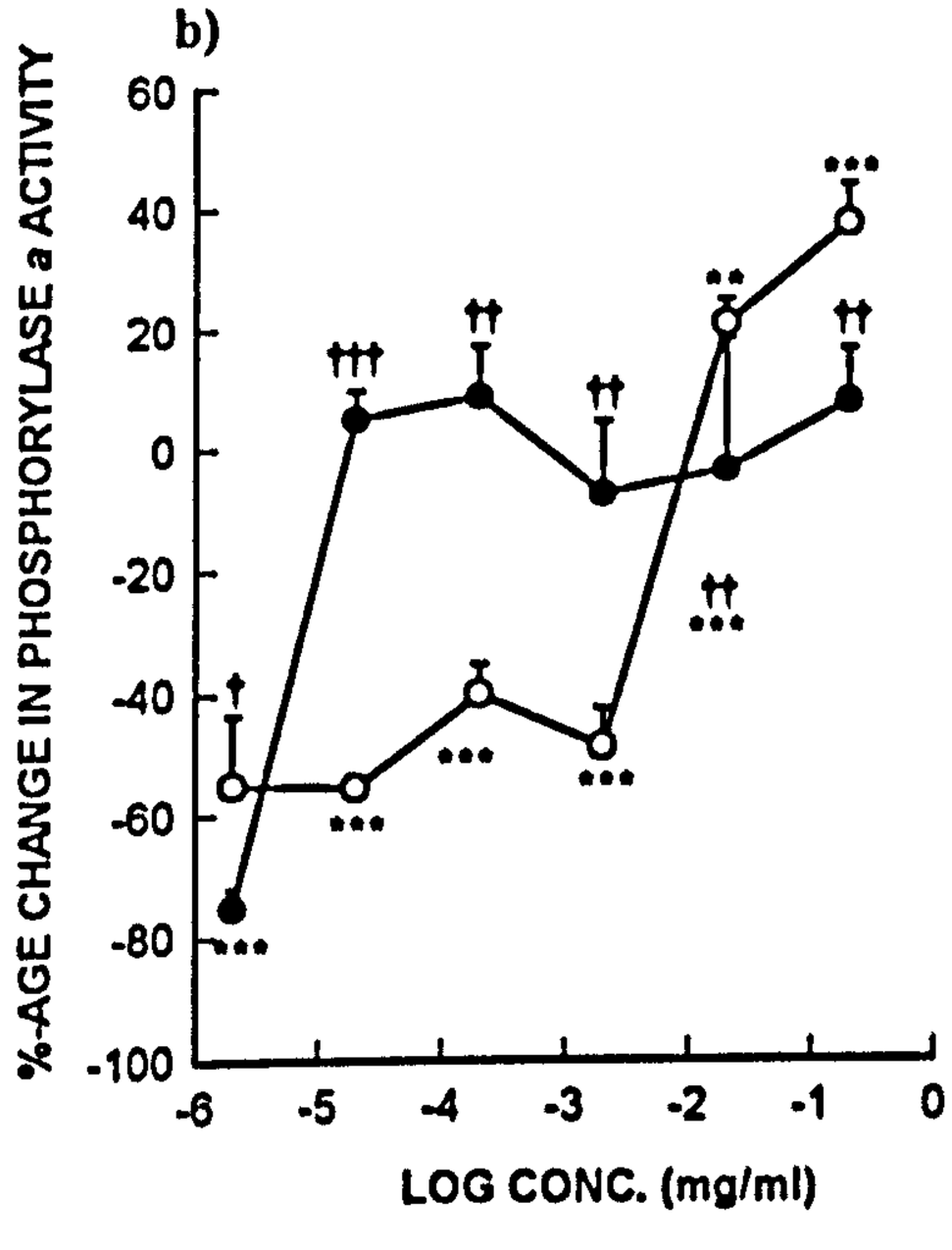
Figure 6.2: Dose-response curves for the effect glucagon and extracts of *Artemisia judaica* (b); *Marrubium vulgare* (c) and *Anvillea carcinii* (d) on glycogen phosphorylase *a* (a) in isolated rat hepatocytes preincubated for 30 min. in Krebs-Henseleit medium and the effect of 3 min exposure to the extracts and hormone on the enzyme activity measured, compared to basal phosphorylase activity * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$ and † = $p<0.05$, †† = $p<0.01$, ††† = $p<0.001$ compared to respective dose of extract alone, p were obtained by ANOVA followed by Dunnet's test.

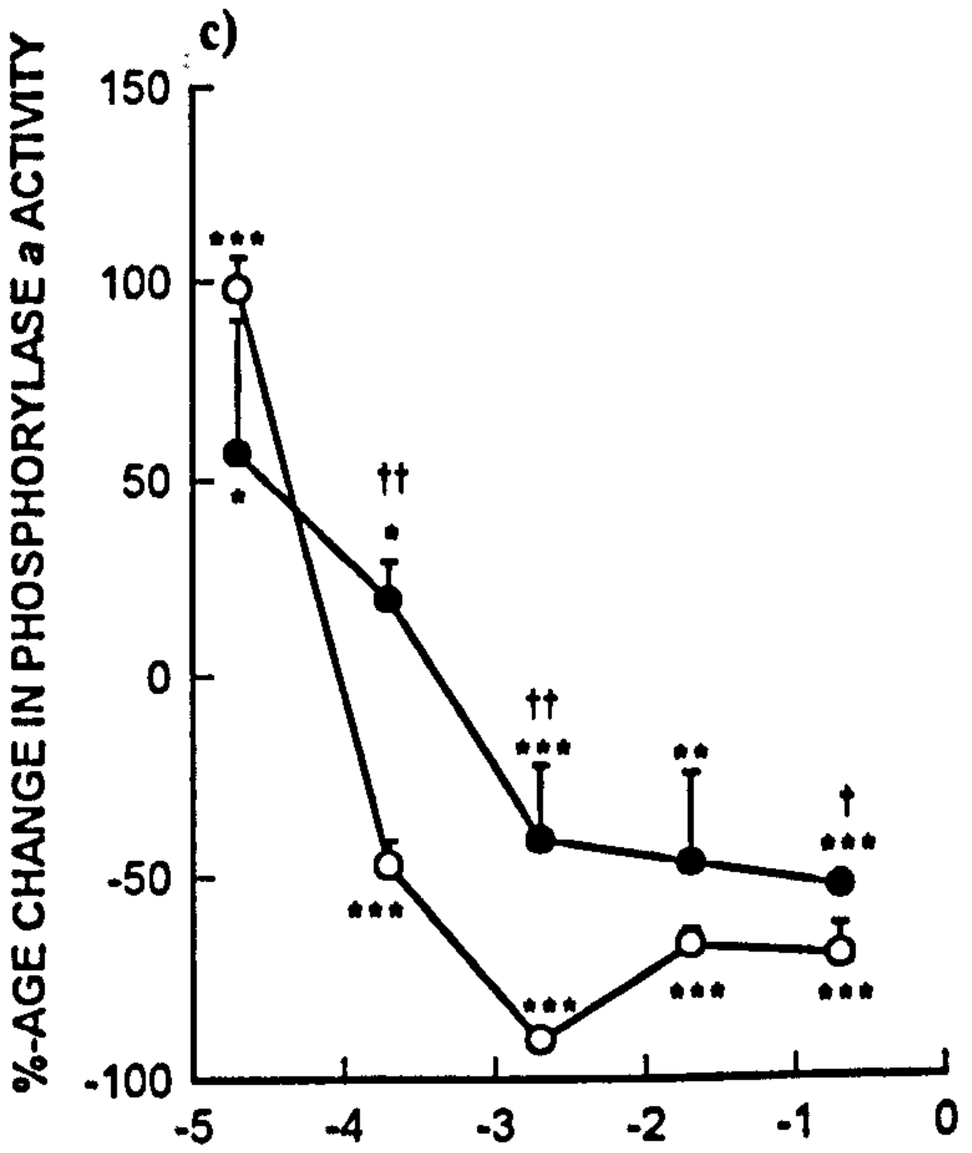
Results are expressed as mean ± s.d. (n=3).

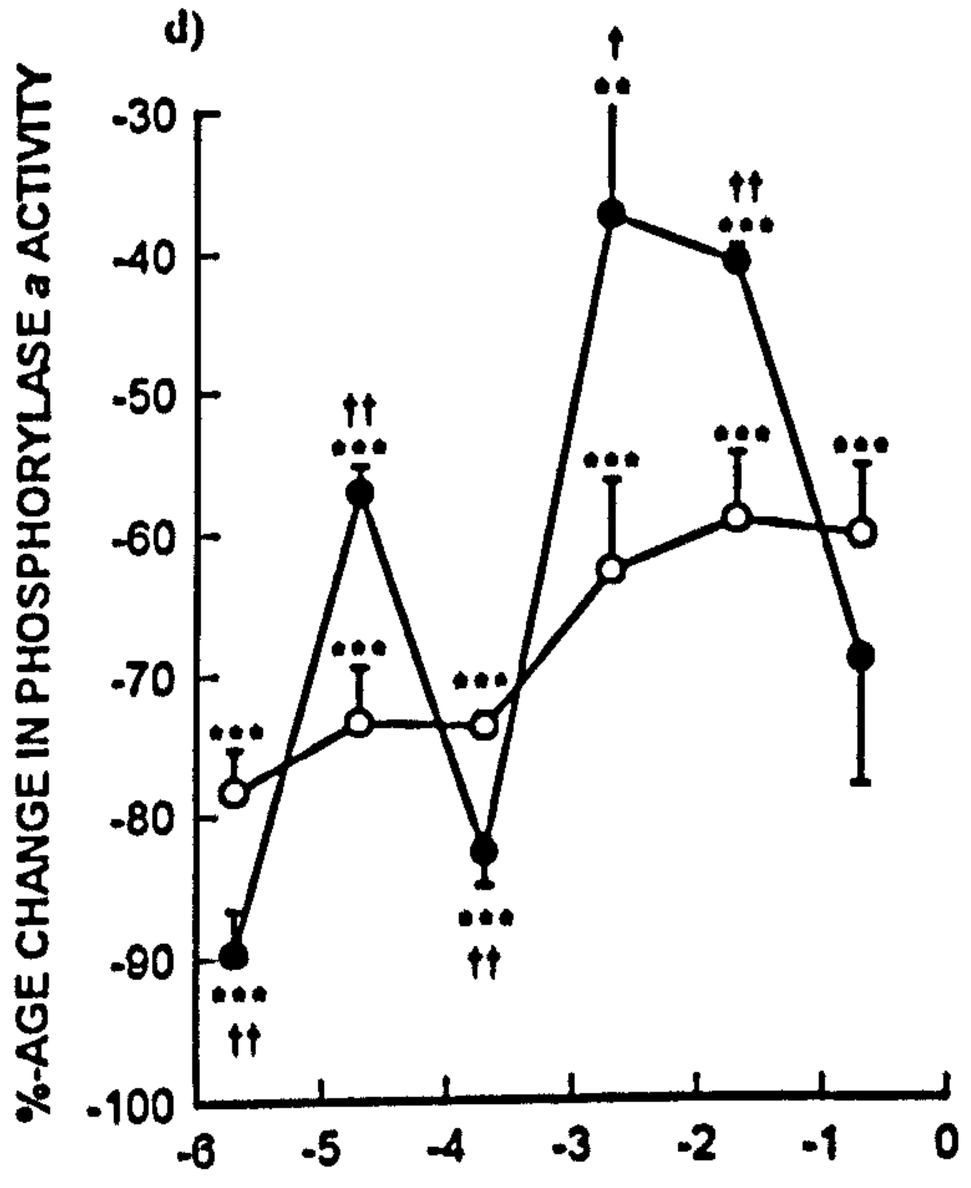
o = Glucagon, *Artemisia*, *Marrubium* & *Anvillea* alone

• = *Artemisia*, *Marrubium* or *Anvillea* + glucagon (10^{-7}M)


a)
%-AGE CHANGE IN PHOSPHORYLASE a ACTIVITY
120
100
80
60
40
20
0
-20
-40
-60
-80
-100
-11
-10
-9
-8
-7
-6
LOG [GLUCAGON] (M)


b)
%-AGE CHANGE IN PHOSPHORYLASE a ACTIVITY
60
40
20
0
-20
-40
-60
-80
-100
-6
-5
-4
-3
-2
-1
0
LOG CONC. (mg/ml)


c)
%-AGE CHANGE IN PHOSPHORYLASE a ACTIVITY
150
100
50
0
-50
-100
-5
-4
-3
-2
-1
0
LOG CONC. (mg/ml)


d)
%-AGE CHANGE IN PHOSPHORYLASE a ACTIVITY
-30
-40
-50
-60
-70
-80
-90
-100
-6
-5
-4
-3
-2
-1
0
LOG CONC. (mg/ml)

that there is a general decrease in enzyme activity except at very high concentration of *Artemisia* (2×10^{-2} g/ml) or low concentrations of *Marrubium* (2×10^{-7}g/ml). This does not fully agree with the results in the previous experiment. The reason for this is unclear but will be discussed later. If the effects of the crude extracts are tested in the presence of glucacon (10^{-7}M), which increases enzyme activity, then all extracts antagonize glucagon action (i.e. the increase in activity expected with 10^{-7}M glucagon is not seen and, indeed, in some cases a marked decrease in activity is observed compared to control levels).

At this stage the crude extract of *Artemisia judaica* was fractionated to give an aqueous (water-soluble) extract and an alcoholic (ethanol-soluble) extract as described in Materials and Methods.

6.3.2 Effect of insulin and glucagon on the activity of glycogen phosphorylase *a* in hepatocytes isolated from normal and STZ diabetic rats.

Tables 6.17 and 6.18 show the effect of different concentrations of insulin and glucagon alone on glycogen phosphorylase *a* in normal and diabetic rats. Insulin did not decrease enzyme activity in hepatocytes isolated from normal or diabetic rats except at 10^{-4}M, which significantly increased the activity ($p<0.001$ & $p<0.01$ in cells from normal and diabetic animals respectively). Under similar conditions, glucagon in different concentrations significantly increased enzyme activity ($p<0.001$) in normal and diabetic rats.

Table 6.16: Controls for experiment of normal and diabetic rat hepatocyte on glycogen phosphorylase *a* activity.

Control	Normal (mean ± s.d)	Diabetic (mean ± s.d.)
Water	31.90 ± 1.66	20.12 ± 0.38
Ethanol	39.26 ± 6.37	40.70 ± 1.14
HCl	15.47 ± 1.04	11.94 ± 0.52
H_2O + HCl	23.69 ± 2.19	17.61 ± 1.43
H_2O + Ethanol	44.81 ± 2.52	36.72 ± 1.02

Table 6.17: Dose-response effect of **insulin** and **glucagon** at concentrations (10^{-4} -10^{-9} M), on glycogen phosphorylase a in isolated **normal** rat hepatocytes compared to control (100%). The cells were preincubated for 30 min in Krebs-Henseleit medium and the effect of 3 min exposure to the hormones on the enzyme activity measured. Values are the mean ± s.d. (n = 6), analysed by ANOVA followed by Dunnet's test. *p<0.05, **p<0.01, ***p<0.001, (* = As compared to control). Basal activity of glycogen phosphorylase a for control (Table 6.16).

%-AGE CHANGE IN PHOSPHORYLASE a ACTIVITY						
Concentration (M)	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10-9
Insulin	123.5±22.6**	15.85±12.3	-4.37±14.5	-20.37±4.82*	3.21±11.1	-11.6±11.5
Glucagon	150.84±6.61***	212.02±17.79***	248.76±27.66***	174.78±15.03***	77.5±11.21**	104.65±16.58**

Table 6.18: Dose-response effect of **insulin** and **glucagon** at concentrations (10^{-4} - 10^{-9} M), on glycogen phosphorylase *a* in isolated **diabetic** rat hepatocytes compared to control (100%). The cells were preincubated for 30 min in Krebs-Henseleit medium and the effect of 3 min exposure to the hormones on the enzyme activity measured. Values are the mean ± s.d. (n = 6), analysed by ANOVA followed by Dunnet's test. *p<0.05, **p<0.01, ***p<0.001, (* = As compared to control). Basal activity of glycogen phosphorylase a for control (Table 6.16)

%-AGE CHANGE IN PHOSPHORYLASE a ACTIVITY						
Concentration (M)	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
Insulin	51.07±4.83**	-7.69±3.64	-1.99±4.75	-8.33±5.75	5.29±19.2	-3.30±9.88
Glucagon	-31.84±12.62*	53.68±2.94**	51.99±6.26**	85.29±15.59**	6.59±20.03	-9.86±8.27

6.3.3 Effect of an aqueous extract of *Artemisia judaica* and insulin on the activity of glycogen phosphorylase a in hepatocytes isolated from normal rat.

The data in Table 6.19 shows the effects of different concentrations of an aqueous extract of *Artemisia* alone or in the presence of different concentrations of insulin on glycogen phosphorylase *a* activity. Incubation of control rat hepatocytes with an aqueous extract of *Artemisia* in different concentrations resulted in decreased glycogen phosphorylase *a* activity at concentrations of extract of 2×10^{-2}, 2×10^{-3}, 2×10^{-5} and 2×10^{-6} g/l (25%, 35%, 37%, and 51% respectively as compared to control ($p<0.01$)) but concentrations of 2×10^{-1} and 2×10^{-4} g/l had no significant effect. The result at 2×10^{-4} g/l looks out of place and may be a false negative as this concentration of extract has shown effects previously (see Figure 6.2b). The highest concentration of *Artemisia* extract has, however, tended to be inactive. When the hepatocytes were incubated with different concentrations of aqueous extract of *Artemisia* with insulin (10^{-6}M), a significant decrease was found at concentrations of 2×10^{-1} g/ml ($p<0.01$), 2×10^{-2} g/ml ($p<0.001$) and 2×10^{-5} g/ml ($p<0.001$) compared to the effects of insulin or *Artemisia* extract alone. At other concentrations, there was no synergistic effect of aqueous extract of *Artemisia* and insulin and, indeed, at 2×10^{-6} g/l *Artemisia* extract, insulin inhibited the activity.

Insulin alone in different concentrations (10^{-9}-10^{-6}M) was less able to decrease glycogen phosphorylase *a* activity in this experiment and this agrees with the results from previous experiments.

In general, a similar pattern of changes in glycogen phosphorylase *a* activity was seen for the other concentrations of insulin used (10^{-9}-10^{-7}M) with synergistic effects being noted (i.e. the effect of insulin plus the aqueous extract of *Artemisia judaica* being more potent than either component alone). It is interesting to note the combinations that have antagonistic effects, however. At the highest concentration of insulin used (10^{-6}M), an antagonistic effect is noted with the most potent concentration of *Artemisia* extract (2×10^{-6} g/l). As the concentration of insulin used falls, the antagonistic effect shifts towards the less potent concentrations of *Artemisia* extract (e.g. insulin 10^{-7}M and *Artemisia* 2×10^{-5} g/l; insulin 10^{-8}M and *Artemisia* 2×10^{-4} g/l and insulin 10^{-9}M and *Artemisia* 2×10^{-3} g/l). The significance of this is unclear at present.

Table 6.19: Dose-response effect of insulin at concentrations (10^{-6} -10^{-9} M) and different concentrations of aqueous extract of *Artemisia* on glycogen phosphorylase *a* in isolated normal rat hepatocyte compared to control (100%). The cells were preincubated for 30 min in Krebs-Henseleit medium and the effect of 3 min exposure to the aqueous extract of *Artemisia judaica* and hormone on the enzyme activity measured. Values are the mean ± s.d (n = 6), analysed by ANOVA followed by Dunnet's test. $^{*,\clubsuit,\#}$ $p<0.05$, $^{**,\clubsuit\clubsuit,\#\#}p<0.01$, $^{***,\clubsuit\clubsuit\clubsuit,\#\#}p<0.001$, (note: * = As compared to control; # = As compared to effect of plant extract alone and ♣ = As compared to effect of insulin). Basal activity of glycogen phosphorylase a for control (Table 6.16).

%-AGE CHANGE IN PHOSPHORYLASE *a* ACTIVITY						
Concentration (mg / ml)	2×10^{-1}	2×10^{-2}	2×10^{-3}	2×10^{-4}	2×10^{-5}	2×10^{-6}
Artemisia	4.76±7.04	-25.8±9.3**	-35.1±9.53**	-9.44±9.44	-37.9±7.21**	-51.25±6.29**
Artemisia + Insulin(10^{-6}M)	-24.7±15.3♣♣♣##	-48.15±5.79♣♣♣##	-34.87±3.12#	-9.61±13.69	-57.59±2.83♣♣♣##	3.41±12.66
Artemisia + Insulin (10^{-7}M)	-36.4±9.1♣♣♣##	-56.37±6.74♣♣♣##	-55.6±5.00♣♣##	-28.67±3.5♣♣	-0.60±10.58♣♣♣##	-54.41±8.95##
Artemisia + Insulin (10^{-8}M)	-82.1±8.77♣♣♣##	-41.28±8.63♣♣##	-56.02±6.70	0.71±2.08	-1.94±1.06♣♣♣	-35.96±27.94##
Artemisia + Insulin (10^{-9}M	-50.09±3.56♣♣♣	-26.29±10.7	0.35±8.09♣♣♣	2.03±8.54♣♣	-55.58±6.40♣♣	-37.03±12.6♣

6.3.4 Effect of an aqueous extract of *Artemisia judaica* and glucagon on the activity of glycogen phosphorylase *a* in hepatocytes isolated from normal and diabetic rat.

Table 6.20 illustrates the significant rise in glycogen phosphorylase a activity when different concentrations of glucagon (10^{-4} - 10^{-9} M) were added to isolated hepatocytes.

As described above, the addition of an aqueous extract of *Artemisia judaica* to the cells caused a significant fall in the activity of glycogen phosphorylase *a* at most concentrations. If glucagon at concentrations of 10^{-6} - 10^{-9} M was added to the cells at the same time as different concentrations of *Artemisia* extract, we found that the effect of the glucagon could be modified. At a concentration of 10^{-6}M, which increases glycogen phosphorylase a by 248% of control, all concentrations of an aqueous extract of *Artemisia* diminished the action of the glucagon though the activity of the glycogen phosphorylase *a* was still significantly higher than their respective control. Aqueous extracts of *Artemisia* had, thus, a partial antagonistic effect on the effect ($p<0.001$ at all concentrations). Similar effects were seen with glucagon at concentrations of 10^{-7} and 10^{-8} M and the same observation was seen in hepatocytes isolated from STZ diabetic rat (Table 6.21).

6.3.5 The effect of an aqueous extract of *Artemisia judaica* and insulin on the activity of glycogen phosphorylase *a* in hepatocytes isolated from STZ diabetic rat.

The effect of *Artemisia judaica* and glucagon on glycogen phosphorylase *a* were further studied by analyzing their effects in hepatocytes isolated from diabetic rats.

Table 6.22 shows that, *Artemisia* extract in the concentrations range 2×10^{-2} to 2×10^{-6} g/ml gave no significant change in the activity but at a concentration of 2×10^{-1} g/ml the activity decreased to 35% of control ($p<0.001$).

The addition of different concentrations of aqueous extract of *Artemisia judaica* to insulin (10^{-6}M) caused a decrease at all concentrations from 2×10^{-1} to 2×10^{-5} g/ml

Table 6.20: Dose-response effect of <u>glucagon</u> at concentrations (10^{-6} - 10^{-9} M) and different concentrations of <u>aqueous</u> extract of *Artemisia* on glycogen phosphorylase *a* in isolated <u>normal</u> rat hepatocyte compared to control (100%). The cells were preincubated for 30 min in Krebs-Henseleit medium and the effect of 3 min exposure to the aqueous extract of *Artemisia judaica* and hormone on the enzyme activity measured. Values are the mean ± s.d (n = 6), analysed by ANOVA followed by Dunnet's test. *,♣, #, $p<0.05$, **,♣♣, ##$p<0.01$, ***,♣♣♣, ##$p<0.001$, (note: * = As compared to control; # = As compared to effect of plant extract alone and ♣ = As compared to effect of glucagon. Basal activity of glycogen phosphorylase a for control (Table: 6.16).

%-AGE CHANGE IN PHOSPHORYLASE *a* ACTIVITY						
Concentration (mg / ml)	2×10^{-1}	2×10^{-2}	2×10^{-3}	2×10^{-4}	2×10^{-5}	2×10^{-6}
Artemisia	4.76±7.04	-25.8±9.3	-35.1±9.44	-9.44±9.44	-37.9±7.21**	-51.25±6.29**
Artemisia + Glucagon (10^{-6}M)	n.d.	153.4±23.3♣♣♣##	144.1±4.31♣♣♣##	155.4±9.30♣♣♣##	90.4±12.4♣♣♣##	3.41±12.66♣♣♣
Artemisia + Glucagon (10^{-7}M)	55.02±3.86♣♣	151.6±25.4♣♣♣##	175.2±14.69♣♣♣	103.1±12.4♣♣♣##	114.4±8.42♣♣♣##	-54.41±8.95##
Artemisia + Glucagon (10^{-8}M)	41.3±8.09♣♣♣##	81.64±17.5♣♣♣	-0.04±12.6♣♣♣##	-13.16±10.1##	21.76±20.5♣♣♣##	33.14±12.7♣♣♣##

Table 6.21: Dose-response effect of **glucagon** at concentrations (10^{-6} - 10^{-9} M) and different concentration of **aqueous** extract of *Artemisia* on glycogen phosphorylase *a* in isolated **diabetic** rat hepatocyte compared to control (100%). The cells were preincubated for 30 min in Krebs-Henseleit medium and the effect of 3 min exposure to the aqueous extract of *Artemisia judaica* and hormone on the enzyme activity measured. Values are the mean ± s.d (n = 6), analysed by ANOVA followed by Dunnet's test. *,♣, #, $p<0.05$, **,♣♣, ##$p<0.01$, ***,♣♣♣, ##$p<0.001$, (note: * = As compared to control; # = As compared to effect of plant extract alone and ♣ = As compared to effect of glucagon. Basal activity of glycogen phosphorylase a for control (Table: 6.16).

%-AGE CHANGE IN PHOSPHORYLASE *a* ACTIVITY						
Concentration (mg / ml)	2×10^{-1}	2×10^{-2}	2×10^{-3}	2×10^{-4}	2×10^{-5}	2×10^{-5}
Artemisia	-34.9±3.31**	-5.20±5.02	1.19±5.10	0.07±4.30	-2.66±3.48	3.81±2.64
Artemisia + Glucagon (10^{-6}M)	65.3±20.3♣♣♣##	91.7±19.3♣♣♣##	104.1±23.5♣♣♣##	81.2±11.1♣♣♣##	64.99±7.55♣♣♣##	61.62±16.3♣♣♣##
Artemisia + Glucagon (10^{-7}M)	20.0±1.03♣♣♣##	18.89±0.85♣♣♣##	19.35±1.64♣♣♣##	15.68±1.08♣♣♣##	21.11±1.10♣♣♣##	19.35±0.52♣♣♣##
Artemisia + Glucagon (10^{-8}M)	47.5±6.18♣♣♣##	64.8±12.89♣♣♣##	20.4±1.64♣♣♣##	-7.53±10.6♣♣♣##	-12.3±16.4♣♣♣##	19.35±0.52♣♣♣##

Table 6.22: Dose-response effect of insulin at concentrations (10^{-6} - 10^{-9} M) and different concentrations of aqueous extract of *Artemisia* on glycogen phosphorylase *a* in isolated diabetic rat hepatocyte compared to control (100%). The cells were preincubated for 30 min in Krebs-Henseleit medium and the effect of 3 min exposure to the aqueous extract of *Artemisia judaica* and hormone on the enzyme activity measured. Values are the mean ± s.d (n = 6), analysed by ANOVA followed by Dunnet's test. $^{*,\clubsuit,\#}$ $p<0.05$, $^{**,\clubsuit\clubsuit,\#\#}p<0.01$, $^{***,\clubsuit\clubsuit\clubsuit,\#\#}p<0.001$, (note: * = As compared to control; # = As compared to effect of plant extract alone and ♣ = As compared to effect of insulin or glucagon. Basal activity of glycogen phosphorylase a for control (Table:6.16).

%-AGE CHANGE IN PHOSPHORYLASE *a* ACTIVITY						
Concentration (mg / ml)	2×10^{-1}	2×10^{-2}	2×10^{-3}	2×10^{-4}	2×10^{-5}	2×10^{-6}
Artemisia	-35.94±3.31**	-5.20±5.02	1.19±5.10	0.07±4.30	-2.66±-3.48	3.81±2.64
Artemisia + Insulin(10^{-6}M)	-9.0±9.49♣♣♣	-0.71±13.8	-32.54±5.80♣♣♣##	-11.4±17.22	-16.7±2.34♣♣♣	n.d.
Artemisia + Insulin (10^{-7}M)	-4.56±8.35♣♣♣	-24.59±12.8♣♣	-21.66±11.07♣♣	-25.45±14.9♣♣	-34.32±9.4♣♣♣	-34.3±9.41♣♣♣
Artemisia + Insulin (10^{-8}M)	1.08±15.03♣♣♣	-26.1±6.65♣♣♣#	-9.06±3.98	-17.74±4.07♣♣	-16.16±16.45♣♣	n.d.

except at a concentration of 2×10^{-2} g/ml which had no effect. Maximum effect was seen at a concentration of 3×10^{-3} g / ml (32 % of control). Insulin at a concentration of 10^{-7}M with different concentrations of aqueous extract of *Artemisia judaica* decreased the activity of the enzyme activity significantly more than insulin (10^{-6}M) with different concentrations of aqueous extract. Insulin (10^{-8}M) decreased the activity significantly with concentrations of *Artemisia* extract at 2×10^{-2}, 2×10^{-3} and $20\times^{-5}$ g /ml but no effect at a concentration of 2×10^{-2} and 2×10^{-4} g / ml.

6.3.6 The effect of an ethanolic extract of *Artemisia judaica* and insulin on the activity of glycogen phosphorylase *a* in hepatocytes isolated from normal rat.

The data of Table 6.23 shows that an ethanolic extract of *Artemisia judaica* alone had little effect on glycogen phosphorylase *a* activity except at concentrations of 2×10^{-1} and 2×10^{-3} g/l , which showed a significant fall in activity ($p<0.01$). The ethanolic extract is, thus, less effective than the aqueous extract by itself.

As noticed previously, insulin had little effect on glycogen phosphorylase *a* activity except in high concentration which, in this experiment, activated glycogen phosphorylase *a*.

The addition of insulin (10^{-6}M) together with different concentrations of an ethanolic extract of *Artemisia* gave no significant change in glycogen phosphorylase *a* activity except at the lowest concentration of *Artemisia* extract (2×10^{-5} g/l) where a reduction was seen which was statistically significant ($p<0.001$). Neither insulin nor *Artemisia* extract at these concentrations gave any significant reduction and, thus, this indicates a synergism of effect at these concentrations on the activity of glycogen phosphorylase *a*. The effect of the ethanolic extract of *Artemisia* with 10^{-7}M insulin was to significantly decrease glycogen phosphorylase *a* activity ($p<0.001$) at all concentrations of the ethanolic extract, the maximum decrease (63% of control) was seen at the high concentration .

Insulin (10^{-8} and 10^{-9}M) together with different concentrations of the ethanolic extract of *Artemisia* gave a similar effect to that seen for insulin (10^{-7}M) with marked synergistic action of the two additions.

Table 6.23: Dose-response effect of glycogen phosphorylase a to <u>insulin</u> at concentrations (10^{-6} - 10^{-9} M) and different concentrations of <u>ethanolic</u> extract of *Artemisia* in isolated <u>normal</u> rat hepatocyte compared to control (100%). The cells were preincubated for 30 min in Krebs-Henseleit medium and the effect of 3 min exposure to the aqueous extract of *Artemisia judaica* and hormone on the enzyme activity measured. Values are the mean ± s.d (n = 6), analysed by ANOVA followed by Dunnet's test. $^{*,\clubsuit,\#}$ $p<0.05$, $^{**,\clubsuit\clubsuit,\#\#}p<0.01$, $^{***,\clubsuit\clubsuit\clubsuit,\#\#}p<0.001$, (note: * = As compared to control; # = As compared to effect of plant extract alone and ♣ = As compared to effect of insulin or glucagon. Basal activity of glycogen phosphorylase a for control (Table:6.16).

%-AGE CHANGE IN PHOSPHORYLASE *a* ACTIVITY					
Concentration (mg / ml)	2×10^{-1}	2×10^{-2}	2×10^{-3}	2×10^{-4}	2×10^{-5}
Artemisia	-19.84±8.12**	-4.84±7.36	-13.51±3.61**	-4.98±8.76	-9.55±4.78
Artemisia + Insulin(10^{-6}M)	-6.29±7.13♣	-7.18±7.35	-13.24±3.54	-12.36±4.88	-30.51±4.71♣♣♣#
Artemisia + Insulin (10^{-7}M)	-63.2±1.7♣♣♣##	-11.30±5.55##	-27.85±5.96♣♣♣	-35.37±6.9♣♣♣##	-23.31±3.00♣♣♣
Artemisia + Insulin (10^{-8}M)	-61.4±3.1♣♣♣##	-21.42±6.91♣♣##	-35.64±4.8♣♣♣##	-45.53±7.9♣♣♣##	-13.15±8.2♣♣♣##
Artemisia + Insulin (10^{-9}M	-41.4±3.2♣♣♣##	-62.5±3.17♣♣♣##	-37.8±6.45♣♣♣##	-34.23±3.6♣♣♣##	-37.1±3.65♣♣♣##

6.3.7 Effect of an ethanolic extract of *Artemisia judaica* and insulin on the activity of glycogen phosphorylase *a* in hepatocytes isolated from STZ diabetic rat.

In the same set of cells isolated from diabetic rats, the effect of an ethanolic extract of *Artemisia judaica* and insulin on glycogen phosphorylase *a* was studied (Table 6.24). Ethanolic extract of *Artemisia judaica* at 2×10^{-1} g /ml alone and with different concentrations of insulin range (10^{-6} to 10^{-8}M) and ethanolic extract of *Artemisia judaica* (2×10^{-2} g/ml) with insulin (10^{-6}M and 10×10^{-8} M) decreased the enzyme activity to 49%, 73%, 27%, 67%, 48% and 23% respectively compared to control ($p<0.001$). The addition of different concentrations of insulin with different concentrations of aqueous extract of *Artemisia judaica* (2×10^{-3} to 2×10^{-6} g / ml) had little effect on the activity of glycogen phosphorylase *a*.

6.3.8 Effect of an ethanolic extract of *Artemisia judaica* and glucagon on the activity of glycogen phosphorylase *a* in hepatocytes isolated from normal and STZ diabetic rat.

As noticed above, glucagon again exhibited a significant activation of glycogen phosphorylase *a* in normal and diabetic at all concentrations (see Tables 6.17 & 6.18). The ethanolic extract of *Artemisia* alone had little effect on glycogen phosphorylase *a* activity but completely counteracted the effects of glucagon on the activity of glycogen phosphorylase *a* at all concentrations ($p<0.001$) in normal and diabetics (Table 6.25 & 6.26).

6.3.9 The effects of artemisinin and santonin on the activity of glycogen phosphorylase a in hepatocytes isolated from normal rat.

As shown in Figures 6.3 and 6.4, artemisinin and santonin had no significant effects alone on glycogen phosphorylase a activity in isolated rat hepatocytes except for a marked increase in activity with artemisinin at high concentrations (10^{-4}M) and some small increases with santonin at various concentrations. Treatment of cells with

artemisinin and insulin (10^{-7}M) gave a marked increase in glycogen phosphorylase *a* activity at concentrations above 10^{-7}M. Artemisinin, however, does appear to inhibit the action of glucagon (10^{-7}M) at all concentrations. The action of santonin on the effect of insulin (10^{-7}M) is rather complex giving a stimulation of glycogen phosphorylase *a* activity at low concentrations (10^{-9}-10^{-8}M) and an inhibition at higher concentrations (10^{-4}M). Santonin at higher concentrations (10^{-5}-10^{-4}M) also seemed to inhibit glucagon action on glycogen phosphorylase *a* activity.

Table 6.24: Dose-response effect of glycogen phosphorylase a to insulin at concentrations (10^{-6} - 10^{-9} M) and different concentrations of ethanolic extract of *Artemisia* in isolated diabetic rat hepatocyte compared to control (100%). The cells were preincubated for 30 min in Krebs-Henseleit medium and the effect of 3 min exposure to the aqueous extract of *Artemisia judaica* and hormone on the enzyme activity measured. Values are the mean ± s.d (n = 6), analysed by ANOVA followed by Dunnet's test. $^{*,\clubsuit,\#}$ $p<0.05$, $^{**,\clubsuit\clubsuit,\#\#}p<0.01$, $^{***,\clubsuit\clubsuit\clubsuit,\#\#}p<0.001$, (note: * = As compared to control; # = As compared to effect of plant extract alone and ♣ = As compared to effect of insulin. Basal activity of glycogen phosphorylase a for control (Table: 6.16).

%-AGE CHANE PHOSPHORLASE *a* ACTIVITY						
Concentration (mg / ml)	2×10^{-1}	2×10^{-2}	2×10^{-3}	2×10^{-4}	2×10^{-5}	2×10^{-6}
Artemisia	-49.1±6.15**	-1.99±2.35	12.25±3.05**	-1.97±5.39	-16.23±7.65**	-18.2±7.54**
Artemisia + Insulin(10^{-6}M)	-73.5±2.3♣♣♣##	-48.0±2.57♣♣♣##	7.25±3.01♣	-4.59±3.00	-14.77±17.1##	-7.28±5.75♣♣
Artemisia + Insulin (10^{-7}M)	-27.9±5.86♣♣♣	-8.93±3.43♣♣	-5.04±4.33♣♣♣	-3.82±5.19	-2.85±5.72	n.d.
Artemisia + Insulin (10^{-8}M)	-67.16±2.7♣♣♣##	-23.6±5.67♣♣♣##	-7.58±3.54♣♣♣	1.51±3.86	2.56±2.07♣♣♣	n.d.

Table 6.25: Dose-response effect of glycogen phosphorylase a to glucagon at concentrations (10^{-6} - 10^{-9} M) and different concentrations of ethanolic extract of *Artemisia* in isolated normal rat hepatocyte compared to control (100%). The cells were preincubated for 30 min in Krebs-Henseleit medium and the effect of 3 min exposure to the aqueous extract of *Artemisia judaica* and hormone on the enzyme activity measured. Values are the mean ± s.d (n = 6), analyzed by ANOVA followed by Dunnet's test. $^{*,\clubsuit,\#}p<0.05$, $^{**,\clubsuit\clubsuit,\#\#}p<0.01$, $^{***,\clubsuit\clubsuit\clubsuit,\#\#}p<0.001$, (note: * = As compared to control; # = As compared to effect of plant extract alone and ♣ = As compared to effect of glucagon. Basal activity of glycogen phosphorylase a for control (Table:6.16).

%-AGE CHANGE IN PHOSPHORYLASE *a* ACTIVITY					
Concentration (mg / ml)	2×10^{-1}	2×10^{-2}	2×10^{-3}	2×10^{-4}	2×10^{-5}
Artemisia	-19.84±8.12**	-4.84±7.36	-13.51±3.61**	-4.98±8.76	-9.55±4.78
Artemisia + Glucagon(10^{-6}M)	-61.5±2.9♣♣♣##	-33.47±3.2♣♣♣##	-13.24±3.54	-12.36±4.88	-30.51±4.7♣♣♣##
Artemisia + Glucagon (10^{-7}M)	-70.1±3.6♣♣♣##	-40.4±3.0♣♣♣##	-15.4±5.46##	-31.89±4.8♣♣♣##	-27.3±1.2♣♣♣##
Artemisia + Glucagon(10^{-8}M)	-71.8±1.8♣♣♣##	-20.4±6.61♣♣##	-32.9±2.24♣♣♣##	-19.6±4.0♣♣##	-41.2±5.38♣♣♣##
Artemisia + Glucagon (10^{-9}M	-52.7±3.0♣♣♣##	-29.4±2.46♣♣♣##	-22.97±3.4♣♣♣##	-24.88±0.7♣♣♣##	-51.5±3.04♣♣♣##

Table 6.26: Dose-response effect of glycogen phosphorylase a to glucagon at concentrations (10^{-6} - 10^{-9} M) and different concentrations of **ethanolic** extract of *Artemisia* in isolated **diabetic** rat hepatocyte compared to control (100%). The cells were preincubated for 30 min in Krebs-Henseleit medium and the effect of 3 min exposure to the aqueous extract of *Artemisia judaica* and hormone on the enzyme activity measured. Values are the mean ± s.d (n = 6), analysed by ANOVA followed by Dunnet's test. *,♣, #, $p<0.05$, **,♣♣, ## $p<0.01$, ***,♣♣♣, ## $p<0.001$, (note: * = As compared to control; # = As compared to effect of plant extract alone and ♣ = As compared to effect of glucagon. Basal activity of glycogen phosphorylase a for control (Table: 6.16).

%-AGE CHANGE IN PHOSPHORYLASE *a* ACTIVITY

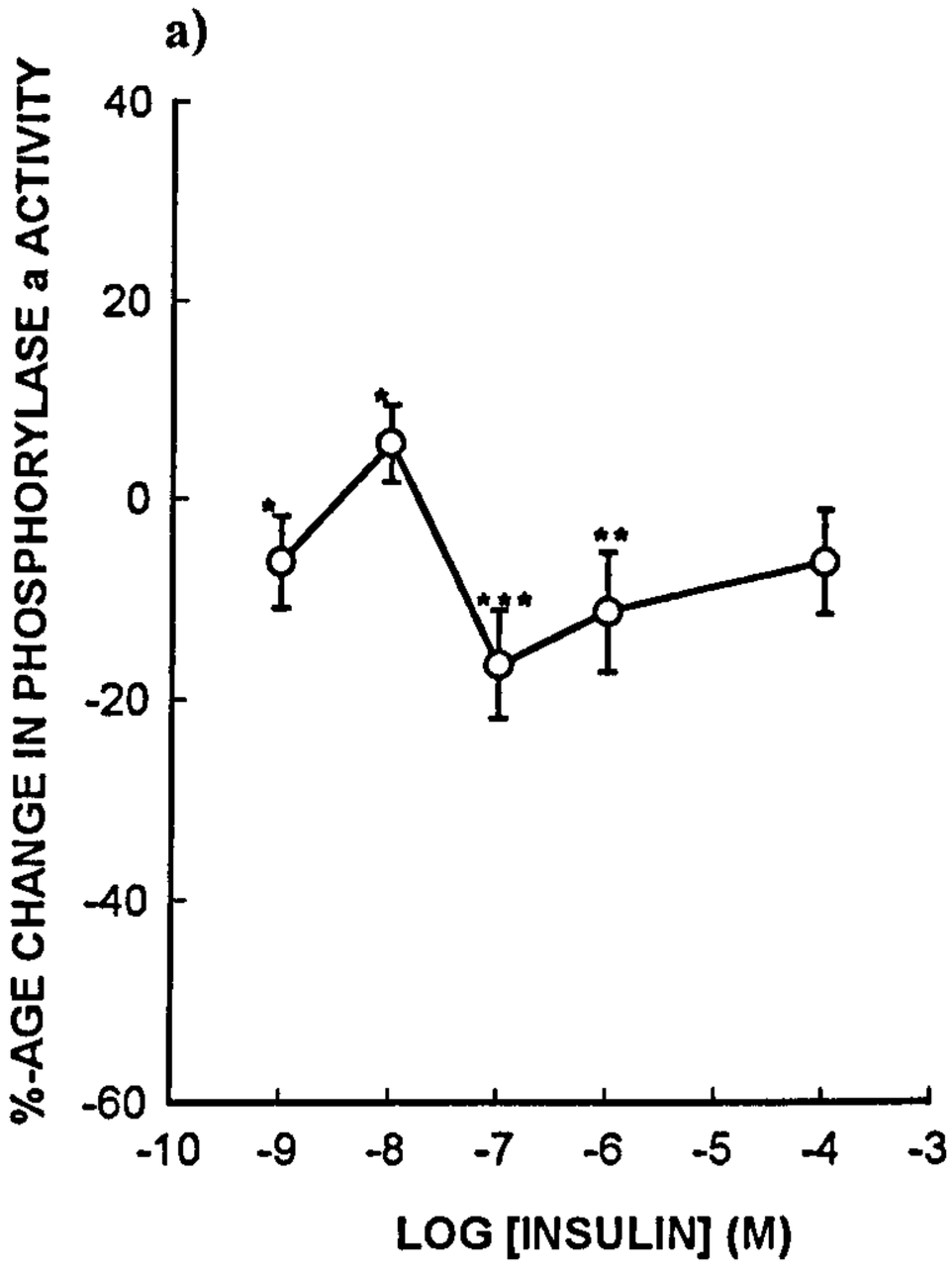
Concentration (mg / ml)	2×10^{-1}	2×10^{-2}	2×10^{-3}	2×10^{-4}	2×10^{-5}	2×10^{-5}
Artemisia	-49.1±2.35**	-1.99±2.35	12.25±3.05	-1.97±5.39	-16.2±7.65*	-18.2±7.54
Artemisia + Glucagon (10^{-6}M)	-75.5±3.2♣♣♣##	-35.76±4.6♣♣♣##	-24.4±2.57♣♣♣##	-20.1±2.77♣♣♣##	-10.2±3.81♣♣♣##	-18.1±3.78♣♣♣##
Artemisia + Glucagon (10^{-7}M)	-71.4±3.1♣♣♣##	-10.66±6.6♣♣♣##	-17.5±5.6♣♣♣##	-38.8±3.65♣♣♣##	-33.69±6.77♣♣##	-3.1±2.34♣♣♣##
Artemisia + Glucagon (10^{-8}M)	n.d.	-11.29±6.17♣♣##	-18.8±3.37♣♣♣##	6.49±8.42##	1.33±5.52♣♣♣##	1.79±5.17♣♣♣##

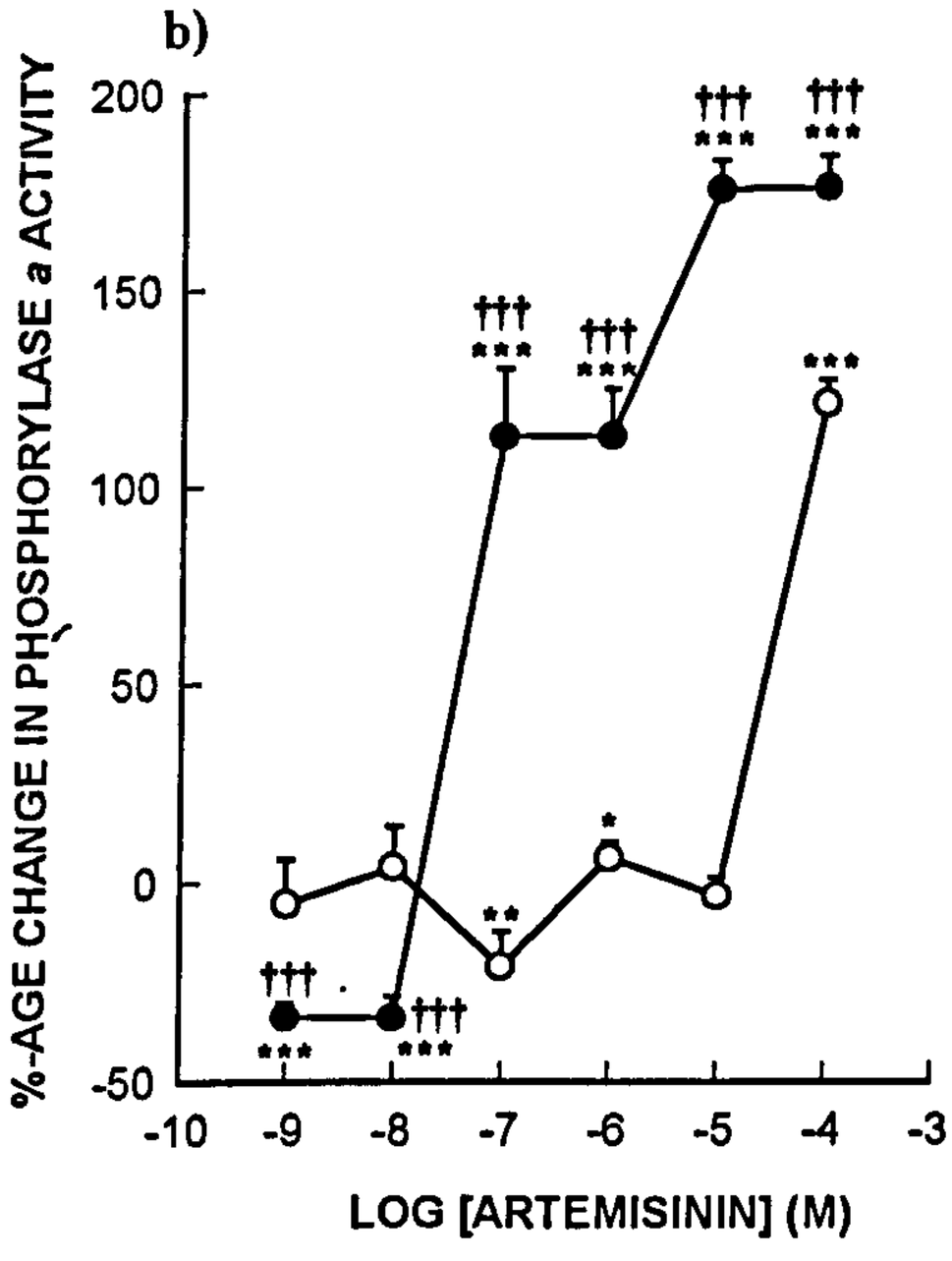
Figure 6.3 Dose-response curves of glycogen phosphorylase *a* to insulin (a); artemisinin (b) and santonin (c) in isolated rat hepatocytes preincubated for 30 min in Krebs-Henseleit medium and the effect of 3 min exposure to the compounds and hormone on the enzyme activity measured, compared to basal phosphorylase activity * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$ and † = $p<0.05$, †† = $p<0.01$, ††† $p<0.001$ compared to respective dose of extract alone, p values were obtained by ANOVA followed by Dunnet's test.

Results are expressed as mean ± s.d. (n=6).

° = Insulin, Artemisinin or santonin alone

• Artemisinin or santonin + insulin (10^{-7}M).


a)
%-AGE CHANGE IN PHOSPHORYLASE a ACTIVITY
40
20
0
-20
-40
-60
-10
-9
-8
-7
-6
-5
-4
-3
LOG [INSULIN] (M)


b)
%-AGE CHANGE IN PHOSPHORYLASE a ACTIVITY
200
150
100
50
0
-50
-10
-9
-8
-7
-6
-5
-4
-3
LOG [ARTEMISININ] (M)

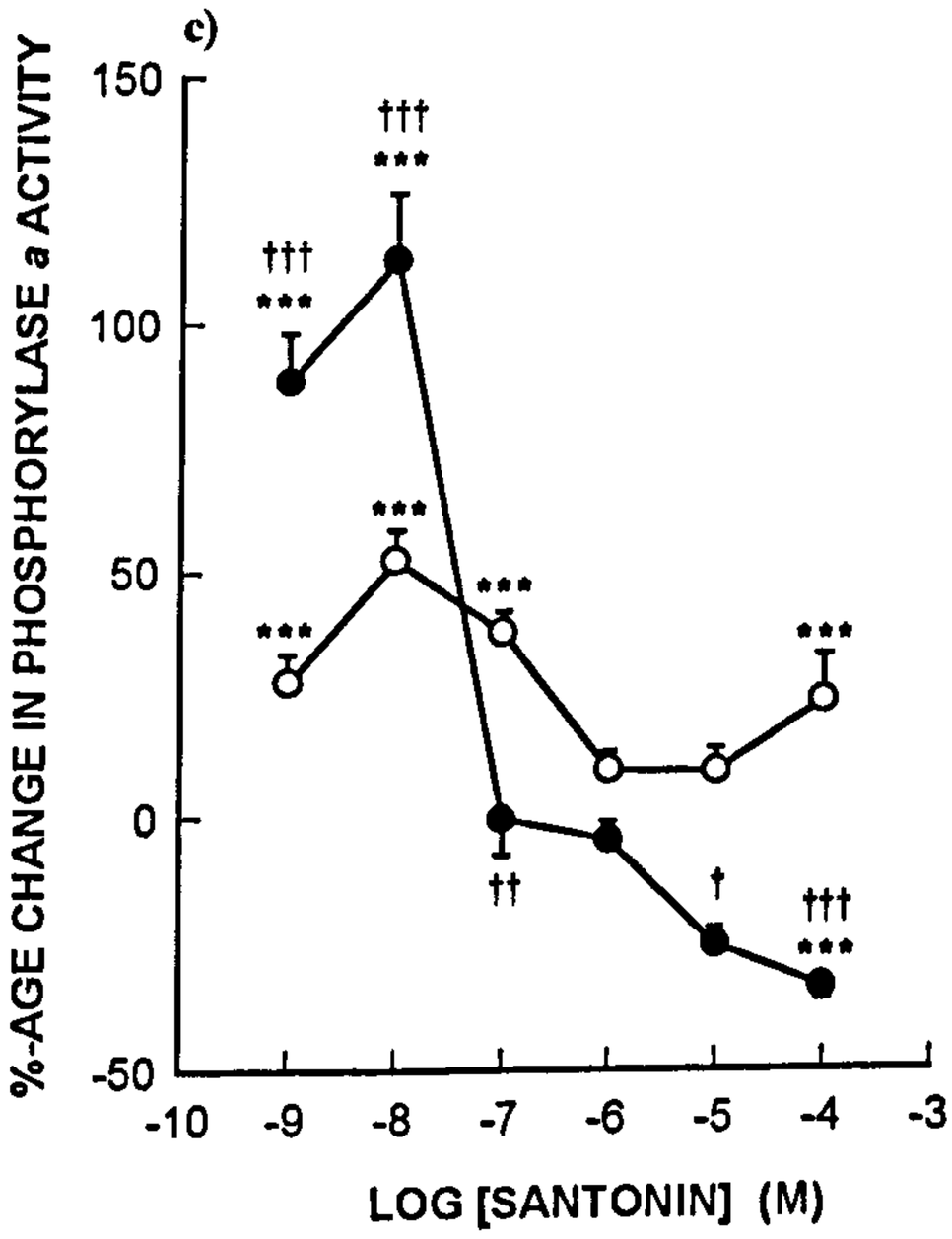
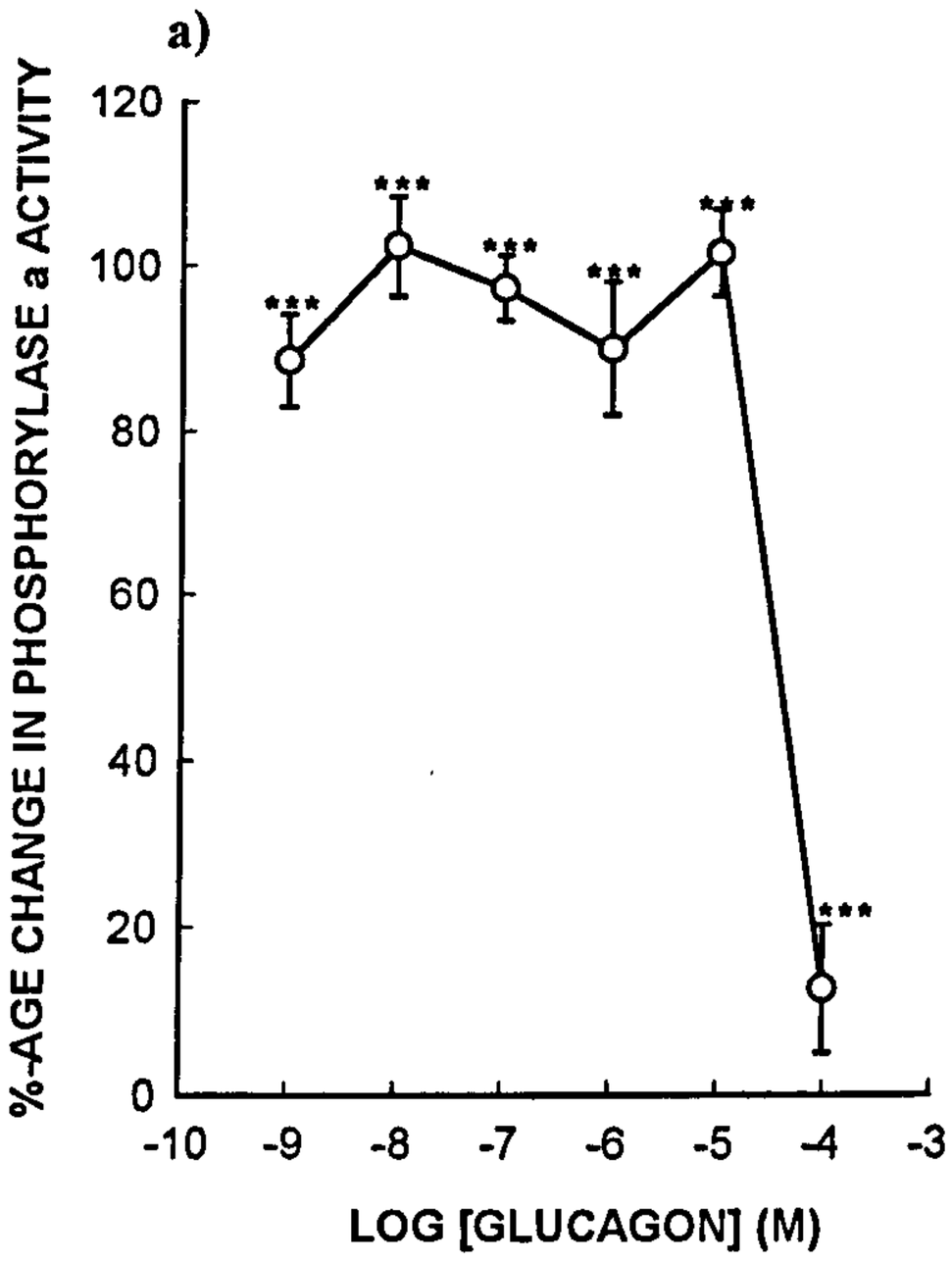

c)
%-AGE CHANGE IN PHOSPHORYLASE a ACTIVITY
150
100
50
0
-50
-10
-9
-8
-7
-6
-5
-4
-3
LOG [SANTONIN] (M)

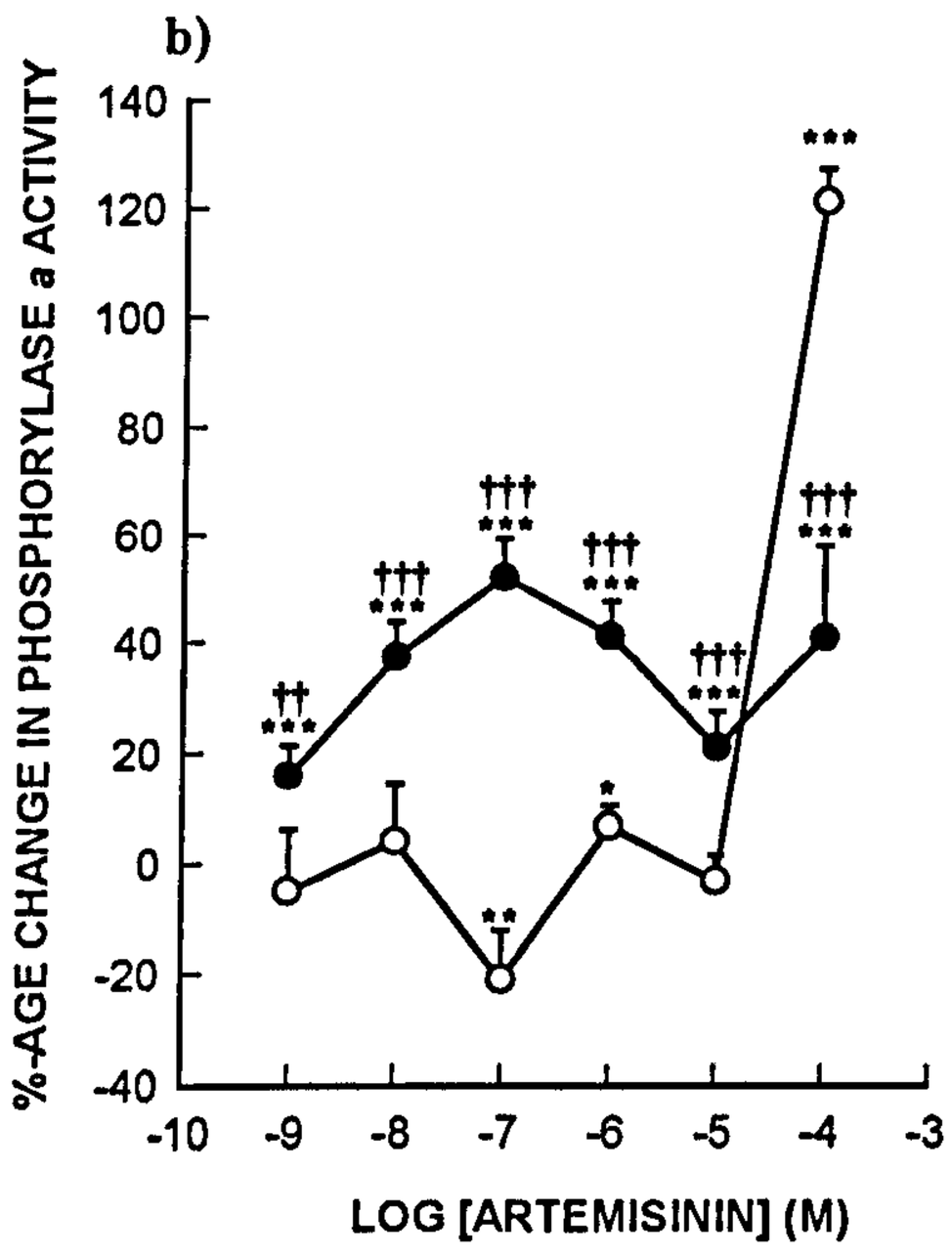
Figure 6.4 Dose-response curves of glycogen phosphorylase *a* to glucagon (a); artemisinin (b) and santonin (c) in isolated rat hepatocytes preincubated for 30 min in Krebs-Henseleit medium and the effect of 3 min exposure to the compounds and hormone on the enzyme activity measured, compared to basal phosphorylase activity * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, and † = $p<0.05$, †† = $p<0.01$, ††† = $p<0.001$ compared to respective dose of extract alone. P values were obtained by ANOVA followed by Dunnet's. Test.

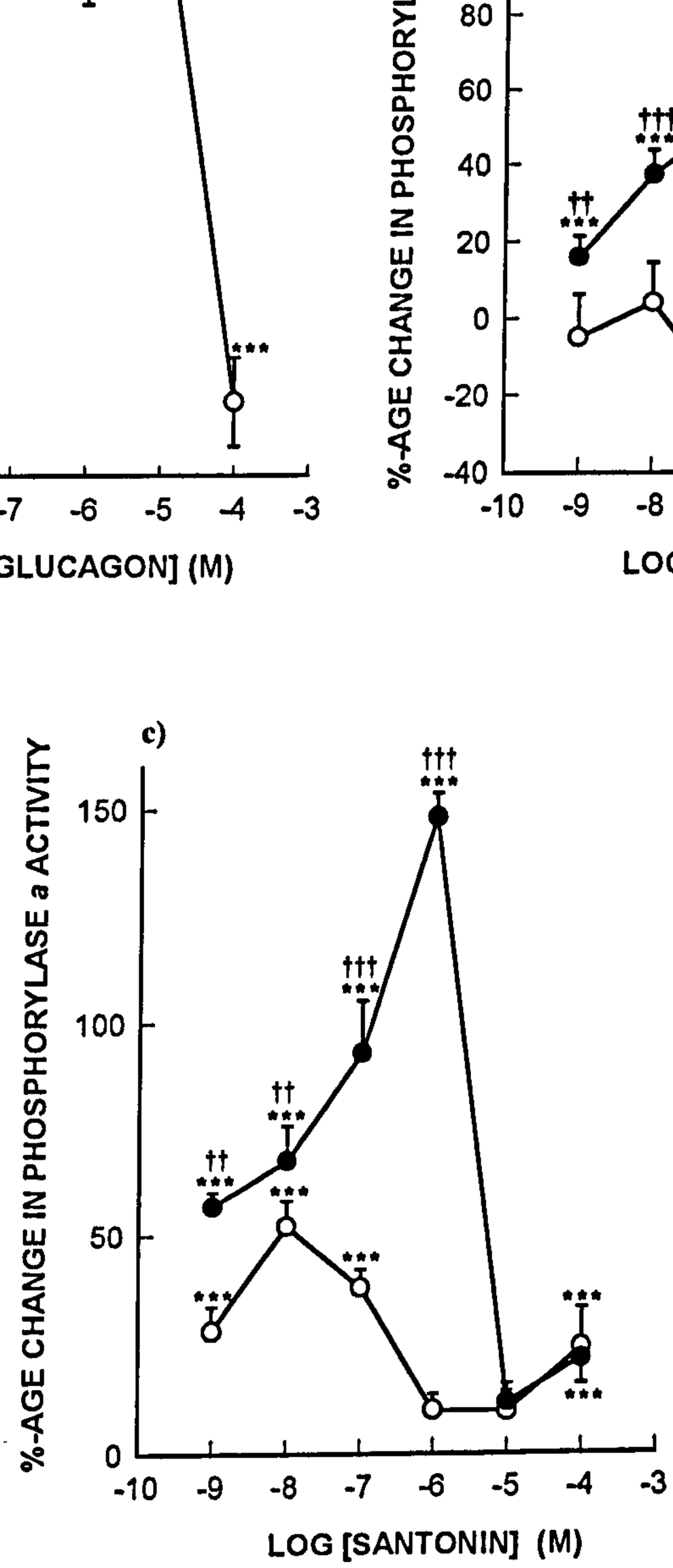
Results are expressed as mean ± s.d. (n=6).

° = Insulin or Artemisinin or santonin

• Artemisinin or santonin + insulin (10^{-7}M).


a)
%-AGE CHANGE IN PHOSPHORYLASE a ACTIVITY
120
100
80
60
40
20
0
-10
-9
-8
-7
-6
-5
-4
-3
LOG [GLUCAGON] (M)


b)
%-AGE CHANGE IN PHOSPHORYLASE a ACTIVITY
140
120
100
80
60
40
20
0
-20
-40
-10
-9
-8
-7
-6
-5
-4
-3
LOG [ARTEMISININ] (M)


c)
%-AGE CHANGE IN PHOSPHORYLASE a ACTIVITY
150
100
50
0
-10
-9
-8
-7
-6
-5
-4
-3
LOG [SANTONIN] (M)

6.4 Discussion:

The liver is an important site for insulin and glucagon action in the regulation of mammalian glucose homeostasis. Liver glycogen phosphorylase *a* is the rate-limiting enzyme of glycogenolysis and exists in two forms as an active *a* form and inactive *b* form (Hems & Whitton, 1980). The present investigation looked at the effects of extracts of *Artemisia judaica*, *Anvillea carcin*ii, and *Marrubium vulgare*, which are widely used in Libyan folk medicine for the treatment of diabetes mellitus, on glycogen phosphorylase *a* activity in hepatocytes isolated from control male rats to see if any of the plants contained substances which could mimic insulin or counteract the effects of glucagon on this activity.

We found that insulin in different concentrations when incubated with control hepatocytes produced little change in glycogen phosphorylase *a* activity. This result is in agreement with reports suggesting that insulin alone has little effect on glycogen phosphorylase *a* activity in liver (Andrew *et al.*, 1985). A crude extract of *Artemisia judaica* alone decreased glycogen phosphorylase *a* activity but when insulin was added together to crude extract, the activity of glycogen phosphorylase *a* was decreased more than with the extract of *Artemisia* alone. The same observations were seen with crude extracts of *Marrubium vulgare* or *Anvillea carcinii*. These three medicinal plants, thus, contain (a) substance(s) which can act synergistically with insulin to decrease glycogen phosphorylase *a* activity in the liver. This could account for some of the proposed hypoglycaemic actions of these plants.

Although the mechanism of action of the biguanides (e.g. metformin) is not completely understood, the presence of insulin is required for metformin to exert many of its effects (Hermann, 1979; Lucis, 1983). The plant extracts could, therefore, be said to show a biguanide-like action, potentiating the action of insulin. *In-vivo*, the blood glucose lowering capacity of metformin does not result from an increase in insulin secretion, but is rather attributed to a combination of actions in peripheral insulin target tissues involving both enhanced glucose metabolism and improved insulin action (Shen & Bressier, 1977). Several *in-vivo* and *in-vitro* studies have pointed out one possible mode of action of the drug at the cell surface - its ability to increase insulin binding to its receptor in various cell types like erythrocytes (Holle *et al.*, 1981), rat hepatoma cells

(Goldfine *et al.*, 1984), human breast cells (Pezzino *et al.*, 1982) and rat adipocytes (Fantus & Brosseau, 1986). Can the plant extracts tested here affect insulin binding? A question for a future study. Recent studies have also demonstrated that metformin improves insulin-mediated [^{14}C]-glucose incorporation into glycogen in cells isolated from the liver and this may represent one of the mechanisms by which biguanides exerts their hypoglycemic action (Melin *et al.*, 1990), So biguanides are known to improve the effect of insulin in some types of diabetes and the presence of insulin is required for the biguanides to exert some of their hypoglycemic action (Muntoni, 1974). Insulin alone, in several reports, has shown little effect on glycogen synthase or phosphorylase *a* in liver cells (Andrew *et al.*, 1985). The extracts of *Artemisia judaica, Anvillea carcinii* and *Marrubium vulgare* may, thus, have a similar mechanism of action to the biguanides although the extracts can have some effect in the absence of insulin unlike the biguanides.

With respect to the action of glucagon on glycogen phosphorylase *a* activity, it is seen that glucagon alone significantly increased glycogen phosphorylase *a*. However, when glucagon was added with the crude extracts of *Artemisia judaica, Anvillea carcinii* and *Marrubium vulgare,* the effect of glucagon was completely abolished. The plant extracts, thus, contain (a) substance(s) which antagonises glucagon action on glycogen phosphorylase *a* activity in isolated hepatocytes. This again could account for some of their hypoglycaemic actions.

Glucagon activates glycogen phosphorylase *a* through an elevation of intracellular cAMP levels, which leads to the activation of a cAMP-dependent kinase and activation of phosphorylase kinase (Sutherland & Robinson, 1979). Insulin on the other hand reduces the glucagon-stimulated elevation of cAMP and activation of phosphorylase (Exton *et al.*, 1971; Siddle *et al.*, 1973). There is general agreement that insulin is effective in antagonizing the action of certain glycogenolytic hormones such as glucagon and alpha$_1$-adrenergic agonists (Andrew *et al.*, 1985). It can therefore be inferred from the data on glycogen phosphorylase a that the crude extracts of *Artemisia judaica, Anvillea carcinii* and *Marrubium vulgare* may have insulin-like activity *in-vitro* and perhaps some of the same actions as biguanides as discussed above.

As previously discussed, we found that the crude extract of *Artemisia* contains a substance which reacts in the glucose assay but is unlikely to be glucose itself. If we

consider the mechanism of action of glucose in liver (Stalmans *et al* 1990), we find that it activates glycogen synthase and inhibits glycogen phosphorylase by the following steps: (1) glucose binds to glycogen phosphorylase as a better substrate, the glycogen phosphorylase is dephosphorylated and inactivated (Carabaza *et al.*, 1992). (2) The dephosphorylation of the phosphorylase releases the inhibitory effect of active phosphorylase on the hepatic phosphatase which now is able to activate glycogen synthase (Carabaza *et al.*, 1992). Glucose is thus a physiological regulator of hepatic glycogen metabolism that promotes inactivation of glycogen phosphorylase (Stalmans *et al.*, 1974) and acts synergistically with insulin (Martin, *et al.*, 1991). Could the crude extract of *Artemisia* have the same action as glucose? What component of the extract gives the effects? These are questions requiring an answer.

If a first step at a fractionation is performed and the crude extract is split into an aqueous and an alcoholic fraction as described in Materials and Methods, then it is seen that the aqueous fraction can enhance the effects of insulin but is poor at inhibiting the actions of glucagon whereas the alcoholic fraction completely abolishes glucagon action and enhances to a certain extent insulin action. The glucagon-inhibiting action of the extract, thus, resides primarily in the ethanol-soluble fraction whereas the insulin-enhancing substance(s) are split between the water- and ethanol-soluble fractions, although more, perhaps, in the water-soluble fraction. The water-soluble fraction has recently been shown to contain inositol-like sugars (Professor.J.Connolly, Department of Chemistry, University of Glasgow; personal communication) but the exact nature of these is not known. This would fit in with the detection of a glucose-like substance in our assays and the effects of glucose on glycogen phosphorylase *a* activity discussed above.

Two compounds, artemisinin and santonin, known to be found in extracts of *Artemisia judaica* (Abdalla *et al.*, 1987), also showed activity in our assay but the activity was the opposite of that seen with the crude extract - an increase in glycogen phosphorylase *a* activity in the presence of insulin. Santonin alone also increased glycogen phosphorylase *a* activity. It is unlikely, therefore, that either of these known components of *Artemisia judaica* can account for the effects seen.

7. General discussion:

Non-insulin-dependent diabetes mellitus is one of the most frequent metabolic disorder in world. As the WHO study group stated in 1985: Its impact is often not fully acknowledged and should attract more attention from health care professionals and planners. Patient with this disease often do not receive optimal care resulting in impaired quality of life, excess mortality and enormous health care costs. So the causes, effects and treatment of diabetes mellitus have been the subject of a great deal of scientific research over the years but despite this there is still controversy over them and the condition and its implications are still not fully understood. Sulphonylureas or biguanides have been widely used for treatment of NIDDM over the past 30 years. In addition to their stimulatory effect on insulin secretion, these compounds seem to possess extra pancreatic effects (Prato *et al.*, 1991) and this has raised questions over their effectiveness in treating symptoms other than the hyperglycaemia, which has resulted in a failure to reduce mortality associated with diabetes. Insulin therapy should not be used in NIDDM patient so long as blood glucose can be maintained within the individual target range by correction of overweight, diet and or exercise but short-term insulin therapy may be necessary in some NIDDM patients during periods of illness. It is clear, therefore, that current treatments of diabetes mellitus, particularly of the non-insulin-dependent type are not ideal and involve drugs that have no defined mechanism of action. Even the apparently straightforward treatment of insulin-dependent diabetes mellitus with insulin is not without its drawbacks such as the continual necessity for injections of the hormone. Clearly work needs to continue to find alternative treatments for these conditions that have a clearer mechanism of action and are easier to administer. Arabic folk medicine contains a number of herbal extracts said to combat the symptoms of diabetes mellitus, chief amongst these are extracts *of Artemisia judaica, Marrubium vulgare* and *Anvillea carcinii.* Little is known of their actions, however, other than empirical work suggesting a reduction in serum glucose levels on taking the extracts. Interestingly, these treatments are aqueous infusions of the herbs taken by mouth and, thus, could represent a source of novel therapeutic agents for the treatment of diabetes mellitus that are easier to take for the patient.

The study of diabetes is made complex by the fact that neither the *in-vivo* nor *in-vitro* animal models can provide a true representation of the range of the human diabetic conditions. The model used here, the STZ-induced diabetic rat, has been put forward as an animal model of IDDM in humans although STZ is known to produce some NIDDM symptoms such as pronounced insulin resistance in the target tissues. Such a model may provide some evidence of action of drugs against both non-insulin- and insulin-dependent diabetes mellitus.

In order to study the potential of drugs to combat diabetes mellitus and to obtain a handle on the possible mechanisms of action of the drugs, it is necessary to look for processes that are controlled by the hormones involved in the development of the disease - the most likely candidates being insulin and glucagon. Many processes of intermediary metabolism are controlled to a greater or lesser extent by these hormones including carbohydrate, protein and lipid metabolism. The major symptom of diabetes mellitus is, however, a raised serum glucose level and, thus, carbohydrate metabolism could be considered as the most important regulatory process. One enzyme involved in carbohydrate metabolism is glycogen phosphorylase *a*, a key enzyme in the regulation of glycogen metabolism, has been shown to be decreased in diabetic rats (Miller et al. 1986). In STZ-diabetic liver, phosphorylase (*a* & total activities) were significantly lower than those seen in the normal animals. cAMP-dependent protein kinase and phosphorylase kinase activities also show substantial reduction during the fasted to refed transition (Pugazhenthi & Khandelwal, 1991) but the enzyme activities were restored to control values by insulin therapy (Roester & Khandelwal, 1987, Stude & Ganeas, 1989). In an *in vitro* study it was clearly demonstrated that, in hepatocytes cultured in serum-free medium, insulin decreased the activity of glycogen phosphorylase (Hartmann, et al. 1987, Lawrence & Zhag, 1994) Glycogen phosphorylase *a* activity is, thus, an insulin-sensitive enzyme activity and can be used to measure insulin-like activity. In this study, extracts of *Artemisia judaica, Marrubium vulgare* and *Anvillea carcinii* were examined for their potential anti-diabetic effects in the STZ-diabetic rat model, on isolated rat hepatocytes using glycogen phosphorylase a activity and metabolism of androst-4-ene-3,17-dione (two insulin-sensitive enzyme systems) and in diabetic human subjects. Time restraints meant that most work was concentrated on *Artemisia judaica*. The extract of *Artemisia judaica* was give to the animal in their

drinking water, body weight, urine volume and urine glucose and food and fluid intake were monitored daily. In normal rat treated with *Artemisia judaica* food and fluid intake and body weight were not changed by 11 days treatment with extract of *Artemisia* but urine volume was found to increase suggesting that *Artemisia judaica* itself acting as a mild diuretic which may be due to the presence of a glucose-like substance in the urine of normal animals following exposure to *Artemisia* extract in the first 6 rats. This may be the substance in the *Artemisia* extract that reacts in the glucose assay which is not reabsorbed by kidney. Assay of *Artemisia* extract gives glucose readings but the nature of the actual substance is unknown at present.

In diabetic rats treated with *Artemisia judaica*, diabetes was induced by administration of STZ and was associated with the characteristic development of hyperglycaemia, hyperphagia, polydipsia and loss of body weight and after treatment with *Artemisia judaica* the urine glucose level was decreased. The decrease in the presence of ketones suggested that there is an improvement in the handling or catabolism of lipids, both of which are beneficial in diabetes. From the results of the present study it can be said that the extract exhibits significant anti-diabetic effects when administered orally. Similar results were obtained from the small clinical evaluation performed in this project.

Artemisia extract treatment of the diabetic animals reversed or partially reversed the effects of diabetes on aniline, aminopyrine and androst-4-ene-3,17-dione metabolism. The addition of *Artemisia* extract directly to microsomes gave some unexpected results with a significant increase in aminopyrine N-demethylation and androst-4-ene-3,17-dione metabolism indicating that some of the effects of the *Artemisia* extract may be direct on the microsomal enzymes whereas the other effects may be indirect (e.g. that on aniline metabolism). It is reported that the diabetic condition, probably via generation of ketone bodies, can elevate aniline metabolism. The fact that *Artemisia* treatment reverses this effect suggests again that *Artemisia* contains a substance or mixture of substances that reverse the perturbation of intermediary metabolism brought about by diabetes mellitus.

Overall the *in vivo* experiments show that crude aqueous extracts *of Artemisia judaica* had significant anti-diabetic properties both when measured as effects on serum glucose and ketone bodies and when examining the changes in xenobiotic and steroid

metabolism known to occur in diabetes mellitus. These results suggested that it was worth examining the possible mechanism of action of these extracts to try and ascertain how they achieved their effects.

It is, however, very difficult to examine the mechanism of action of drugs at the whole animal level and, thus, a better, more controllable system was sought. One such system that has been well studied in this laboratory when looking at the action of hormones is the isolated rat hepatocyte model. Here hepatocytes are separated from the liver and kept in suspension or culture for up to 12 days. Potentially active substances can be added to the cells at precise concentrations and for precise periods of time, thus allowing a greater control of the experimental conditions. By measuring the activity of some insulin-sensitive enzymes, it should be possible to examine if the herbal extracts have any insulin-like activity. To this end glycogen phosphorylase a and androst-4-ene-3,17-dione metabolism were examined in isolated hepatocytes treated with the various plant extracts.

In the present study *Artemisia judaica*, *Anvillea carcinii* and *Marrubium vulgare* were used and tested for their ability to decrease glycogen phosphorylase *a* in normal and streptozotocin-induced diabetes rat hepatocytes as a measure of their insulin-like action. In hepatocytes isolated from normal rats, a crude extract of *Artemisia judaica*, *Anvillea carcinii* and *Marrubium vulgare* inhibited the activity on glycogen phosphorylase *a* but when insulin was added together with crude extracts of *Artemisia judaica*, *Anvillea carcinii* and *Marrubium vulgare*, the activity of glycogen phosphorylase a was decreased more than with the extracts alone, These three plants, thus, contain (a) substance(s) which can act synergistically with insulin to decrease glycogen phosphorylase *a* activity in the liver. If the same experiments are repeated with hepatocytes isolated from diabetic rats then it is seen that the cells are less responsive to hormones and to the plant extracts although some synergistic effects of insulin and the plant extracts are still seen.

If the crude extract of *Artemisia judaica* is sub-fractionated by extraction with ethanol, it is seen that, of the two fractions, the water-soluble fraction retains insulin-like actions whilst the alcohol-soluble fraction shows a marked glucagon antagonistic action and little insulin-like activity. There are, thus, at least two active substances in the crude extract of *Artemisia judaica*. More work needs to be done here to further fractionate

the extract and eventually arrive at an active fraction with one (or a limited number) of constituent(s).

It is seen that glucagon-inhibiting action of the extract, thus, residues primarily in the ethanolic-soluble fraction, whereas the insulin-enhancing substance(s) are split between the water-and ethanolic-soluble fractions, which suggested future work to continue by trying to purify the active ingredients from the aqueous and ethanolic extracts.

The results from the androst-4-ene-3,17-dione assay were disappointing given that Hussin & Skett. (1987) had previously shown a marked effect of insulin on these enzymes. No effect of insulin, *Artemisia judaica* and insulin with *Artemisia* extract was seen on any steroid metabolisms enzyme activity studied and, indeed, a significant decrease was seen with *Artemisia judaica* and insulin. Changing the preincubation period, changing the incubation medium, testing different types of insulin with extract and altering the weight and age of the animals gave no different results. We did not find any positive results with *Artemisia* extract or the other plant extract, *Marrubium vulgare* and *Anvillea carcinii* in different concentration and at different times. The very limited clinical studies possible during this time period were encouraging showing that the crude extract of *Artemisia judaica* appeared to reduce the amount of insulin needed to maintain a steady serum glucose level and, in some cases, could replace more conventional anti-diabetic treatment. Much more in the way of clinical studies, including double-blind testing of plant extracts would be necessary, however, before any definite conclusions could be drawn about the potential of these plant extracts to be used as treatments of diabetes mellitus.

In summary, this project has shown that traditional herbal treatments of diabetes mellitus in use in Libya today (and over many years) do have some basis in scientific fact. The extracts do appear to be beneficial in diabetic patients as claimed and do have some anti-diabetic effects in a rat model system of diabetes mellitus although the results are not as striking in this study as they were in a previous unpublished study from this laboratory. On examining some insulin-sensitive enzymes in the isolated rat hepatocytes, it was found that extracts of *Artemisia judaica, Marrubium vulgare* and *Anvillea carcinii*, all contained substances that either mimicked insulin, antagonised glucagon or acted synergistically with insulin in affecting the enzymes studied. The extracts, thus,

have many possible ways in which they could be acting as anti-diabetics, remembering that these studies only involved the examination of liver enzymes.

Future work must concentrate in a number of areas; one, isolation and identification of the active principle(s) of the extracts, two, closer investigation of the mechanism of action of the extracts on the liver to give anti-diabetic effects including where in the cells the substances act (at the receptor, at the level of the second messenger(s) or directly on the target enzymes) and three, do these extracts have any potential anti-diabetic effects on any other insulin- or glucagon-sensitive tissue. A start has been made to examine these potentially life-saving herbal plants but a great deal more needs to be done before they can, perhaps, realise their potential as a new breed of treatments for diabetes mellitus.

REFERENCES

8. REFERENCES

Abdalla, S.S. (1986). Effects of the crude extract of the medicinal plant *Artemisia judaica L.* on guinea-pig smooth muscles. *Dirasat.* **13,** 115-125.

Abdalla, S.S. & Zarg, M.H.A. (1987). Cirsimaritin, a flavone isolated from *Artemisia judaica* on isolated guinea-pig ileum. *Planta medica.* **53,** 322-324

Agrawal, K. K., Pampori, N.A., & Shapiro, B. H. (1995). Thin-layer chromatographic separation of regioselective and stereospecific androgen metabolites. *Analytical Biochemistry.* **224,** 455-457.

Al-Jibouri, A. (1983). A Cardiophylline in *Artemisia scoparia*. in: Abstracts of the Third *Symposium on Scientific Research, 20-25th Baghdad, Iraq.* 217-218.

Al-Khazraji, S.M., Al-Shamaony, L. A. & Twaij, H.A. (1993). Hypoglycaemic effect of *Artemisia herba alba.* 1. Effect of different parts and influence of solvent on hypoglycaemic activity. *J. Ethnopharm.* **40,** 163-166.

Alwan, A., Al-Gaillany, K. S., & Naji, A. (1989). Inhibition of the binding of 3H-benzo[a] pyrene to rat liver microsomal protein by plant extract. *Int. J. Crude Drug Res.* **1,** 33-37.

Andrew, P., Thomas, P., Martin-Requero, A., & Williamson, J.R. (1985). Interactions between insulin and α-1 adrenergic agents in the regulation of glycogen metabolism in isolated hepatocytes. *J. Biol.Chem.* **260,** 5963-5973.

Bailey, C.J. & Flatt, P.R. (1990). 'Models for testing new hypoglycaemic drugs' in 'New anti-diabetic drugs' ed. Bailley & Flatt, pub. Smith-Gordon & Co. London.

Bailey, C.J. & Nattrass, M., (1988). Non insulin dependent diabetes mellitus. Treatment with metformin. *Clin. Endocrinol. Metabolism.* **2**, 455-76.

Bailey, C.J. & Day, C. (1989). Traditional plant medicines as treatment for diabetes. *Diabetes care*, **12**, 553- 564.

Banhnak, B.R. & Gold, A.H., (1982). Effects of alloxan diabetes on the turnover of rat liver glycogen synthase . *J. Biol. Chem.* **257**, 8775-8780.

Beesj, R., Blackmore, P.F. & Corbin, J. D. (1995). Discriminatory insulin antagonism of stimulatory effects of various cAMP analogous on adipocyte lipolysis and hepatocyte glycogenolysis. *J. Biochemistry.* **260**, 15781-15788.

Berg, A. & Gustafsson, J.A. (1973). Regulation of hydroxylation of 5α-androstane 3α, 17β-diol in liver microsomes from male and female rats. *Journal of Biological Chemistry.* **248**, 6559-6567.

Berry, M. N., Edwards, M. & Barritt, G.F. (1991). Isolated hepatocytes preparation, properties and applications. *Elsevier Science Publishers, Amsterdam.* 460-469.

Bishop, J.S. & Larner, J. (1967). Rapid activation-inactivation of liver uridine diphosphate glucose-glycogen transferase and phosphorylase by insulin and glucagon *in-vivo. J. Biol. Chem.* **242**, 1354-1356.

Blackmore, P.F., Jeannet, F.A, Chan, F.M. & Exton, J. (1979). Studies on α-adrenergic activation of hepatic glucose output. J.*Biol. Chem.*. **254**, 2828-2834.

Bloom, A. & Ireland, J. (1980). A colour atlas of diabetes. Wolf Medical Publications, London, 34-35.

Bone, A.J. (1990). Animal models in diabetic research' in 'Textbook of Diabetes' ed. Pickup J & Williams G. Blackwell Scientific Publications, Oxford.

Bowman W. C. & Rand M. J. (1983). 'Textbook of pharmacology' 2nd edition, Blackwell Scientific publications, Oxford, 614-678.

Brodie, B.B., Axelrod, J., Cooper J.R., Gaudette, L.E., Ladu, B.N., Mitoma, C. & Underfriend, S. (1955). Detoxication of drugs and other foreign compounds by liver microsomes. *Science* **121,** 603-604.

Carabaza, A., Ciudad, C.J., Baque, S. & Guinovart, J.J. (1992). Glucose has to be phosphorylated to activate glycogen synthase, but not to inactivate glycogen phosphorylase in hepatocytes. *FEBS* **296,** 211-214.

Carrol, J.J., Smith, N & Babson, A. L. (1970). A colorimetric serum glucose determination using hexokinase and glucose-6-phosphate dehydrogenase. *Biochem. Med.* **4,** 171-180.

Chawalit, K., Srelarvbsa, P. & Thithapardha, A. (1982). Glucagon and insulin in the control of liver metabolism. *Disphos.* **10,** 81-86.

Clarke, B.F., & Duncan, L.J. (1979). Biguanide treatment in the management of insulin independent (maturity onset) diabetes: Clinical experience with metformin. *Res Clin. For.* **1,** 53-63.

Conney, A.H., Lu Y.H., Somogyi, A., West, S. & Kuntzman, R. (1973). Effect of the cytochrome P-450's. *Drug Metab. Dispos.* **1,** 199-209

Cooper, D.Y., Levin, S.S., Narasimhulu, S., Rosenthal, O. & Estabrook, R.W. (1965). Photochemical action spectrum of the terminal oxidase of the mixed function oxidase system. *Science* **147,** 400-402.

Curnow, R.T., Rayfield, E.J. & George, D.T. (1975). Control of hepatic glycogen metabolism in the monkey: effect of glucose, insulin and glucagon administration. *Am. J. Physiol.* **228,** 80-87

Defeng, W.U. & Arthur, I. (1993). Combined effects of streptozotocin induced diabetes plus 4-methylpyrazole treatment on rat liver cytochrome P-4502E. *Archives of Biochemistry and Biophysics.* **302,** 175-182.

Dent, P., Lavoinne A., Nakielny, S., Caudwell, F.B., Watt, P & Cohen, P. (1990). The molecular mechanism by which insulin stimulates glycogen synthesis in mammalian skeletal muscle. *Nature*. **348**, 302-308.

Dewulf, H. & Hers, H.G. (1968). Mechanism of control of hepatic glycogenesis by insulin. *J. Biol Chem*. **248**, 3483-3488.

Dixon, R. L., Hart, L. G., Rogers, L. A. & Fouts, J. R. (1963). The metabolism of drugs by liver microsomes from alloxan-diabetic rats: Long term diabetes. *Journal of Pharmacology and Experimental Therapeutics.* **142,** 312-317.

Dixon, R. L., Hart, L. G. & Fouts, J. R. (1961). The metabolism of drugs by liver microsomes from alloxan diabetic rats. *Journal of Pharmacology and Experimental Therapeutics*. **133**, 7-11.

Einarsson, K., Gustafsson, J. A., & Stenberg, A. (1973). Neonatal imprinting of liver microsomal hydroxylation and reduction of steroids. *J. Biol. Chem.* **248,** 4987-4997.

Einarsson, K., Gustafsson, J.A., & Hellstrom, K. (1974). Influence of clofibrate on liver microsomal hydroxylation of cholesterol and androstenedione. *Biochem. Pharmacol.* **23,** 13-19.

Eisenbarth, A.S. (1986). Type 1 diabetes mellitus. *N. Engl. J. Med*, **314,** 1360-1368.

Elgadi, A.A. (1989). Usage of some plants in Libyan Folk-Medicine. vol. **2** Libyan Govt. Press, Tripoli 63-64.

Elgadi, A.A. (1990). Usages of some plants in Libyan Folk-Medicine, vol. **1**, Libyan Govt. Press, Tripoli 9-10.

Espinal, J. (1989) 'Understanding Insulin Action' Ellis Horwood Ltd, Chichester 91-98.

Exton, J.H. Lewis, S.B., & Ho, R.J. (1971). The role of cyclic AMP in the interaction of glucagon and insulin in the control of liver metabolism. *Ann. NY. Acad Sci.* **185,** 85-100

Exton, J.H., Robinson, G.A., Sutherland, E.W. & Park, C. R. (1971). Studies on the role of adenosine 3,5-monophosphate in the hepatic actions of glucagon and catecholamines. *Biochem.* **246,** 6166-6177

Fahmy, I.R., Ahmed, Z.F. & Abdul Moniem, F.M. (1958). Santonin an anthelmintmic in *Artemisia herba alba. Bull.De L Institute Du Desert D Egypt.* p 33.

Fantus, I.G. & Brosseau, R. (1986). Mechanism of action of metformin, insulin receptor and postreceptor effects in-vitro and in-vivo. *J Clin. Endocrinol. Metab.* **60,** 989-905.

Farjou, I.B., Al-Anni, M. & Guirges, S.Y. (1987). J. Fac. Med. Baghdad Dept. of Pharmacology, Medical School, Baghdad University, Baghdad, Iraq. **29, 2.**

Farjou, I.B. & Al-Lami, A.D (1988). Effect of *Artemisia* extract on blood glucose and plasma insulin in normal and diabetic rabbits. *J. Fac. Med. Baghdad* **30,** 239-249.

Favreau, L.V. & Schenkman, J.B. (1987). Decrease in the level of a constitutive cytochrome P-450 (RLM5) in hepatic cytochrome of diabetic rats. *Biochem. Biophys. Res. Commun.* **142,** 623-630.

Favreau, L.V., Malchoff, D.M., Mole, J.E. & Schenkman, J. B. (1987). Responses to insulin by two forms of rat hepatic microsomal cytochrome P-450 that undergo major (RLM5b) elevations in diabetes. *J.Biol. Chem.* **262,** 14319-14326.

Ferner, R.E. & Neil, H.A.W. (1988). Sulphonylureas and hypoglycaemia. *Bio. Med. J.* **296,** 949-950.

Galal, E.E., Kandl, A., Abdellatif, M.,Khede, T. & Khafagy, S.M. (1974). Cardiac pharmaco-toxicological studies of judaicin, isolated from *Artemisia judaica. Planta Med.*, **25,** 88-91.

Ganong, W.E. (1989). Review of Medical Physiology 14th edition Prentice-Hall International.

Gibson, G.G. & Skett, P. (1994). Introduction to Drug Metabolism, Chapman and Hall, London, 230-233.

Ginsberg, H. & Ravtiel, E.G. (1981). Effect of insulin therapy on insulin resistance in type II diabetic subjects: evidence for heterogeneity. *Diabetes* **30,** 739-45.

Goldfine, I.J., Iwamoto Y., Pezzino, V. (1984). Effect of biguanides and sulfonylureas on insulin receptors in cultured cells. *Diabetes Care* **7,** 54-58.

Gustafsson, J. & Stenberg, A.D. (1974). Irreversible androgenic programming at birth of microsomal and soluble rat liver enzymes active on androst-4-ene-3,17-dione and 5α-androstane-3α-, 17β-diol. *J. Biol. Chem.* **249,** 711-718.

Gustafsson, J. A. (1973). The formation of 16,17-dihydroxylated C_{19} steroids from 16-dehydro C_{19} steroids in liver microsomes from male and female rats. *Biochemica et Biophysica Acta*, **296,** 179-188.

Harding, B.W., Wong, S.H. & Nelson, D.H. (1964). Carbon monoxide-combining substances in rat adrenal. *Biochim Biophys. Acta.* **92,** 415-417.

Harris, R. A. (1994). Carbohydrate metabolism Major metabolic pathways and their control in "Textbook of Biochemistry," Ed. Devlin, T. M; Wilsy-Liss press, NewYork 291-358.

Hartmann, H., Probst, I., Jungermann, K & Creutzfeldt, W. (1987). Inhibition of glycogenolysis and glycogen phosphorylase by insulin and proinsulin in rat hepatocyte cultures. *Diabetes.* **36,** 551-555.

Hems, D.A. & Whitton, P.D. (1980). Control of hepatic glycogenolysis. *Physiol. Rev.* **60,** 1-23.

Hems, D. A. & Whitton, P.D. (1973). Stimulation by vasopressin of glycogen breakdown and gluconeogenesis in the perfused rat liver. B*iochem. J.* **136,** 705-709.

Hermann, L.S. (1979). Metformin: a review of its pharmacological properties and therapeutic use. *Diabete Metab*. **5,** 233-245.

Hers, H. G. (1976). The control of glycogen metabolism in the liver. *Annu. Rev. Biochem*. **45,** 167-190.

Hoftiezer, V & Carpenter, A. M. (1973). Comparison of streptozotocin- and alloxan-induced diabetes in rat, including volumetric quantitation of the pancreatic islets. *Diabetologia*. **9,** 178-184.

Holle, A, Mangel W, Dreyer, M, Kuhnan, J & Rudiger, H.W. (1981). Biguanide treatment increase the number of insulin receptor sites on human erythrocytes. *N.Engl. J. Med.* **251,** 353-366.

Holle, A., Mangelo, W. & Dreyer, M. (1986). Biguanide treatment increases the number of insulin receptor sites in human erythrocytes. *N. Eng. J. Med.* **305**, 563-568.

Home, P. (1989). 'Editorial' *Diabetic Med.***6**, 3-5

Husni, A.A.,Twai, J. & Ammar Albadr, A. (1988). Hypoglycaemic activity of *Artemisia Herba Alba. J Ethnopharmacology*. **24**, 123-126.

Hussein-Ayoub, S.M. (1990). Antibacterial and antifungal activities of some Libyan aromatic plants. *Planta Med.* **56**, 644-645.

Hussin, A.H. & Skett, P. (1986). Maintenance of steroid metabolism in primary cultures of adult, rat hepatocytes in serum-free medium. *Biochem. Soc. Trans.* **14**, 914-915.

Hussin, A.H. & Skett, P. (1987). The effect of insulin on steroid metabolism in isolated rat hepatocytes. *Biochem.Pharmac.* **36**, 3155-3159.

Hussin, A.H. & Skett, P. (1988). Lack of effect of insulin in hepatocytes isolated from streptozotocin-diabetic male rats. *Biochem.Pharmac.* **37**, 1683-1686.

Ichikawa, Y. & Yamano, T. (1967). Reconversion of detergent- and sulphydryl reagent-produced cytochrome P-420 to cytochrome P-450 by polyols and glutathione. *Biochim. Biophys. Acta.* **131** 490-497.

Joslin, E.P. (1985). 'Diabetes Mellitus', 12th edition, Lea and Febiger, Philadelphia.

Kaji, T., Kaja, K., Miezi, N, Tomohiro, N., Ejiri, N. & Sakurgawa, N. (1990). Possible mechanism of the stimulatory effect of *Artemisia* leaf extract on the proliferation of cultured endothelial cells: Involvement of basic fibroblast growth factor. *Chem. Pharm. Bull.* **38**, 2494-2497.

Kamataki, T., Maeda, K, Yamazoe, Y., Nagai, T. & Kata, R., (1983). Sex difference of cytochrome P-450 in the rat: Purification, characterization and quantitation of constitutive form of cytochrome p-450 from liver microsomes of male and female rats. *Arch. Biochem. Biophys.* **225,** 758-770.

Kato, R. & Gillette, J.R. (1965). Sex differences in the effects of abnormal physiological states on the metabolism of drugs by rat liver microsomes. *J. Pharmac. Exp. Ther.* **150,** 285-291.

Kato, R., & Onoda, K. (1970). Study on the regulation of the activity of drug oxidation in rat liver microsomes by androgen and estrogen. *Biochem. Pharmacol.* **19,** 1649-1660.

Kato, R, Taakanaka, A. & Onoda, K. (1971). Effect of adrenalectomy or alloxan diabetes on the substrate interaction with cytochrome P-450 in the oxidation of drugs by liver microsomes. *Biochem. Pharmacol.* **20,** 447-458.

Kawad, J. (1992). New hypotheses for the mechanisms of streptozotocin and alloxan induced diabetes mellitus. *Yakuqaku Zasshi.* **112,** 773-791.

Kennedy, D.L., Piper, J.M. & Baum, C. (1988). Trends in use of oral hypoglycaemic agents. *Diabetes Care* **11,** 558-562 .

Khafagy, S.M. & Tosson, S., (1968). Crystallographic, optical and chromatographic studies of judaicin, bitter principle of *Artemisia judaica L. Planta med.* **16,** 446-449

Khafagy, S.M., Gharbe, S. A. & Sarg, T. M. (1971). Phytochemical investigation of *Artemisia herba alba. Planta Medica* **20,** 90-97.

Khafagy, S.M., Abdelsalam, M.A. & El-Ghazoly, M.G., (1976). A calorimetric method for the estimation of judaicin bitter principle of *Artemisia judaica. Planta medica.* **30,** 21-24.

Kiso, Y., Ogasawara, N., & Hikino, H., (1984). Antihepatotoxic principles of *Artemisia capillaris* buds. *Planta med.* **50,** 81-85.

Klingenberg, M & Garfinkel, P. (1958). Pigments of rat liver microsomes. *Arch. Biochem. Biophys.* **75,** 376-386.

Krebs, E.G. & Fischer, E.H. (1962). Molecular properties and transformations of glycogen phosphorylase in animal tissues. *Adv. Enzymol.*, **24,** 263-290.

Kreuther, W. & Goldberg, N. D. (1967). Dependence on insulin of the apparent hydrocortisone activation of hepatic glycogen synthase. *Proc. Natl. Acad. Sci. U.S.A.* **58,** 1515-1519.

Kreutzberger A. & Loch M. (1985). Muscle relaxants, IV: Monoacylbutyroguanamines. *Arch.Pharm.* **319,** 289-295.

Kupfer, D. (1980). Endogenous substrates of monooxygenases: Fatty acids and prostaglandins. *Pharmac. Ther.* **11,** 469-496.

Lawrence, C.J. & Zhag, J. (1994). Control of glycogen synthase and phosphorylase by amylin in rat skeletal muscle. Hormonal effects on the phosphorylation of phosphorylase and distribution of phosphate in the synthase subunit. *J. Biol. Chem.* **269,** 11595-11600.

Lebovitz, H.E. (1985). Oral hypoglycemic agents. In The Diabetes Annual-vol. 1. KGMM Allerti and Krall L P Eds. *Amsterdam, Elsevier.* 93-110.

Letoir, L.F. & Cadini, C.E., (1957). Biosynthesis of glycogen from uridine diphosphate glucose. *J.Am. Chem. Soc.* 79, 6340-6346.

Lewis, H.W., (1977). Medical Botany, Plants Affecting Man's Health John Wiley, New York, 820-824.

Lillioja, P. (1988). Impaired glucose tolerance as a disorder of insulin action *N. Eng. J. Med.*, **318**, 1217-1225.

Lowry, O.H., Rosenbrough N.J., Farr A.L. & Randal R.J. (1951) Protein measurement with folin phenol reagent. *J Biochem.*, **193**, 265-275.

Lu, A.Y.H. & West, S.B. (1980). Multiplicity of mammalian microsomal cytochrome P-450 *Pharmacol. Rev.* **31**, 277-295.

Lucis, O.J. (1983). Pharmacological update. The status of metformin in Canada. *Can. Met Assoc. J.* **128**, 24-26.

Luciano,V.S. (1990) 'Human physiology; The mechanisms of body function'. Fourth edition New York St. Louis San Francisco, Auckland, 513-525.

Martin, J.L., Veluaja; Ross, K., Johnson, L.N., Fleet, G.W.J., Ramsden, N.G., Bruce, I., Orchard, M.G. (1991). Glucose analogue inhibitors of glycogen phosphorylase: The design of potential drugs for diabetes. *Biochemistry.* 30, 10101-10116.

Melander, A. (1988). NIDDM treatment with sulfonylureas, in "Clinical Endocrinology and Metabolism", Nattrass, M Hale, P. J., Eds. London, Balliere Tindall. 443-53.

Melin, B. Cherqui, M.J. & Blivet, M. (1990). Dual effect of metformin in cultured rat hepatocytes: Potentiation of insulin action and prevention of insulin-induced resistance *Metabolism.* 39, 1089-1095.

Miller, T. B. & Larner, J. (1973). Mechanism of control of hepatic glycogenesis by insulin. *J. Biochem.* **248**, 3483-3488 .

Miller, T.B. (1978). Phosphorylase activation sensitivity in hearts of diabetic rats. *Am. J. Physiol.* (*Endocrinol. Metab.* 9): E134-E140.

Miller, T.B., Garnache, A.K., Cruz, J. McPherson, R.K. & Wolleben, C. (1986). Regulation of glycogen metabolism in primary cultures of rat hepatocytes. *J. Biol. Chem.* **261,** 785-790.

Mossa J. S. (1985). Phytochemical and biological studies on *Artemisia abyssinica* an antidiabetic herb in Arabian folk medicine. *Fitoterapia.* **56, 311-314.**

Muntoni, S. (1974). Inhibition of fatty acid oxidation by biguanides. Implication for metabolic physiopathology. *Adv. lipid Res.* **12, 311.**

Nash, T. (1953). The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. *J. Biol. Chem.* **55,** 416-422.

Oates, N.S., Shah, R.R. & Smith, R.L. (1981). Phenformin induced lactic acidosis associated with impaired debrisoquine hydroxylation *Lancet* **1, 837-838.**

Oesch, F. & Friedberg, T. (1987). Xenobiotic metabolising enzymes are not restricted to parenchymal cells in rat liver. *Mol. Pharmacol.* **32,** 463-470.

Omura, T. & Sato, R. (1962). A new cytochrome in liver microsomes. *J Biol. Chem.* **237,** 1375-1376.

Omura, T. & Sato, R. (1964). The carbon-monoxide-binding pigment of liver microsomes. I. Evidence for its hemeprotein nature. *J.Biol Chem.* **239, 2370-2378**

Paine, A.J. (1981). Hepatic cytochrome P-450. *Essays in Biochemistry.* **17, 85-121**

Peacock, J. & Tattersall, R.B. (1984). The difficult choice of treatment for poorly controlled maturity onset diabetes: Tablets of insulin. *Br Med J.* **288, 1956-59.**

Pezzino, V., Trischitta, V. & Purello, F. (1982). Effect of metformin on insulin binding to receptors in cultured human lymphocytes and cancer cells. *Diabetologia* **23, 131-135.**

Pugazenthi, S. & Khandelwal, R.L. (1990). Insulin like effects of vanadate on hepatic glycogen metabolism in nondiabetic and streptozotocin-induced diabetic rats. *Diabetes.* **39, 821-827.**

Pugazenthi, S. & Khandelwal, R.L. (1991). Kinases and phosphatases of hepatic glycogen metabolism during fasted to refed transition in normal and streptozotocin-induced diabetic rats. *Biochemistry International* **23, 515-524.**

Reinke, L.A., Stohs, S.J. & Rosenberg, H. (1978). Increased aryl hydrocarbon hydroxylase activity in hepatic microsomes of streptozotocin diabetic female rats. *Xenobiotica* **8,** 769-778.

Roesler, W.J. & Khandelwal, R.L. (1987). Regulation of rat liver glycogen phosphorylase concentration by *in-vivo* relative levels of glucagon and insulin. *Endocrinology.* **121, 227-232.**

Rouer, L.A & Leroux, J.P. (1980). Liver microsomal cytochrome P-450 and related monooxygenase activities in genetically hyperglycaemic and lean streptozotocin treated mice. *Biochem. Pharmacol.* **29,** 1959-1962.

Salhanick, A.L., Konowitz, P. & Amatruda, J.M. (1983). Potentiation of insulin action by a sulphonylurea in primary cultures of hepatocytes in normal and diabetic rats *Diabetes* **32,** 206-212.

Schenkman, J.B. (1990). Induction of diabetes and evaluation of the diabetic state on cytochrome P-450 expression. *Methods in Enzymology.* **206, 325-331.**

Schenkman, J.B., Remmer, O. & Estabrok, R. (1967). Spectral studies of drug interactions with hepatic microsomal cytochrome. *Mol. Pharmacol.* **3, 113-123.**

Seglen, P.O. (1976). Preparation of isolated rat liver cells. *Methods Cell. Biol.* **13,** 29-83

Shen, S.W. & Bressler. R. (1977). Clinical pharmacology of oral antidiabetic agents. *N. Eng. J. Med* **296,** 493-497.

Siddle, K. & Kane-Maquire, B. (1973). The effects of glucagon and insulin on adenosine 3',5'- cyclic monophosphate concentration in an organ culture of mature rat liver. *Biochem.J.* **132,** 765.

Shen, L.C., Villar-Palasi, C. & Laner, J. (1970). Glycogen metabolism and the mechanism of action of cyclic AMP. *Physiol. Chem. Phy.* **2,** 536-541.

Skett, P. (1986). Sex dependent effect of streptozotocin-induced diabetes mellitus on hepatic steroid metabolism in the rat. *Acta Endocrinol.* **111,** 217-221.

Skett, P & Joels, L.A. (1985). Different effect of acute and chronic diabetes mellitus on hepatic drug metabolism in the rat. *Biochemical Pharmacology.* **4,. 287-289.**

Skett, P. Mode, A., Rafter, J., Sahlin, L. & Gustafsson, J.A. (1980). The effects of gonadectomy and hypophysectomy on the metabolism of imipramine and lidocaine by the liver of male and female rats. *Biochemical Pharmacology.* **29,. 2759-2762.**

Skett, P. & Hussin, A.H. (1987). The effect of insulin on steroid metabolism in isolated rat hepatocytes. *Biochemical Pharmacol.* **36, 3155-3159.**

Skett, P. Cochrane R.A. & Joels L.A. (1984). The role of androgens in the effect of diabetes mellitus on hepatic drug metabolism in the male rat. *Acta Endocrinol.*(Copenh.) **107,** 506-512.

Stalmans, W, Bollen, M, Toth, B & Gergely, P. (1990). Short - term hormonal control of protein phosphatases involved in hepatic glycogen metabolism. *Adv. In. Enzym.* **30**, 305-327.

Stalmans, W, Dewulf, H. & Hers, H. G. (1971). The control of liver glycogen synthase phosphatase by phosphorylase, *Eur. J. Biochem. 18*, 582-587.

Stalmans, W. & Hers, H.G. (1973) in '*The enzyme*' (Boyer, P. D., ed.) 3d edn, vol.9, pp.309-361, Academic Press, New York.

Stalmans, W. & Hers, H.G. (1975). The stimulation of liver phosphorylase b by AMP, fluoride and sulfate, A technical note. The specific determination of the a and b forms of liver glycogen phosphorylase. *Eur. J Biochem.*, **54**, 341-350.

Stalmans, W. Dewulf, H. Hue, L. & Her, H.G. (1974). The sequential inactivation of glycogen phosphorylase and activation of glycogen synthase in liver after the administration of glucose to mice and rats. *Eur. J. Biochem.* **41**, 127-134

Steinberg, P., LaFranconi, W.M., Wolf, C.R., Waxman, D.J. & Stowers, J.M. (1972). Oral treatment in diabetes. *Endocrinol. Metab.* **1**, 721-760.

Stenberg, A. (1976). Developmental, diurnal and oestrous cycle-dependent changes in the activity of liver enzymes. *J. Endocr.* **68**, 265-272.

Studer, R.K & Ganas, L. (1989). Effect of diabetes on hormone-stimulated and basal hepatocyte calcium metabolism. *Endocrinology.* **125**, 2421-2433.

Sutherland, E.W. & Robinson, G.A. (1971). The role of cyclic AMP in the control of carbohydrate metabolism. *Diabetes* **18**, 797

Thomas, J., Shlender, K. K. & Larner, J. (1968). A rapid filter paper assay for UDP-glucose glycogen glucosyl-transferase, including an improved biosynthesis of UDP-14C glucose. *Anal. Biochem.* **25,** 486-499.

Toshyuki, K., Kayoko, K., Nsimba, M., Tomohiro, H., Naoko, E., & Nobuo, S. (1990). Possible mechanism of the stimulatory effect of *Artemisia* leaf extract on the proliferation of cultured endothelial cells: Involvement of basic fibroblast growth factor. *Chem. Pharm. Bull.* **38,** 2494-2497.

Twaij, H.A., Elisha, E.E. & Khalid, R.M. (1989). Analgesic studies on some Iraqi medicinal plants. *Int. J. Crude Drug Res.* **2,** 109-112

Van de Werne, G. & Jeanrenaud, B. (1987). Glycogen metabolism: An overview. *Diabetes Metab. Rev.* **3,** 47-78.

Van der Werve, G, Stalman, W. & Hers, H.G. (1977). The effect of insulin on the glycogenolytic cascade and on the activity of glycogen synthase in the liver anaesthetized rabbits. *Biochem. J.* **162,** 143-146.

Watt, J.M. & Breger-Brandwizk, M.G. (1962). The medicinal and poisonous plants of South and Eastern Africa, 2nd Ed. E and S Livingstone, Edinburgh.

Watt, J.M. & Wood, E.D. (1988). Health: Conventional and complementary approaches. London, Ray Soc. Med., WHO Expert Committee: Diabetes Mellitus second report Geneva, World Health Org., 1980 tech. Rep. Ser. 646.

Waxman, D.J., Morrissey, J. J. & Leblanc, G.A. (1989). Female-predominant rat hepatic P-450 forms J(IIE1 and 3(IA1) are under hormonal controls distinct from those of the sex-specific P-450 forms. *Endocrinology* **124,** 2954-2966.

Weiner, M., Buterbaugh, G.G. & Blake, D.A. (1972). Inhibition of hepatic drug metabolism by cyclic 3',5'-adenosine monophosphate. *Res. Commun. Chem. Pathol. Pharmacol.* **3**, 249-263.

Witters, L. & Avrucb, J. (1978). Insulin regulation of hepatic glycogen synthase and Phosphorylase. *Biochemistry*. **17**, 406-410.

WHO Expert Committee: Diabetes Mellitus. 2nd rep. Geneva, World Health Org., 1980 Tech.rep. ser. 646)

GLASGOW UNIVERSITY